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PREFACE

The basic scientific aspects of the transplantation of tissues from man to man and from one animal to another are being actively studied in many research centers. This monograph on Histocompatibility Testing presents the research results discussed at the recent Conference and Workshop on the subject held at the National Academy of Sciences building in Washington, D.C., and at the Duke University Medical Center. It is believed that histocompatibility testing will assist in the selection of more suitable tissue donors and thereby result in an increased percentage of successful transplants.

The Division of Medical Sciences of the National Academy of Sciences–National Research Council is indebted to the Committee on Tissue Transplantation for organizing and carrying out the Conference and Workshop. Drs. Russell, Amos, Koprowski, Trentin, and Winn, acting for the Committee, arranged the program. Drs. Russell, Amos, and Winn also served as the editors of this monograph. Acknowledgement is also given for the financial support, furnished by the organizations listed on the preceding page, which made it possible to gather together the participants.

CONFERENCE AND WORKSHOP

ON

HISTOCOMPATIBILITY TESTING

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PART I.

Conference on Histocompatibility Testing

Introductory Remarks

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This conference on Histocompatibility Testing is a natural sequence to the conference on Human Kidney Transplants sponsored by the Committee on Tissue Transplantation of the National Academy of Sciences-National Research Council and held in this same room on 26-27 September 1963. At that conference the results of the world-wide efforts in clinical renal homotransplantation were thoroughly reviewed (Transplantation 2:147–165 and 581–600, 1964).

Among the more significant themes running through the presentations and discussions was the clear fact that long-term success in human renal transplantation is more likely when the donor and recipient are either parent and child, or siblings. This might well have been anticipated since the importance of the genetic relationship between donor and recipient has long been recognized as the fundamental principle in transplantation immunology. Nevertheless, the great vulnerability of skin grafts, and probably grafts of most tissues, to destruction when transferred between mammals of relatively minor genetic diversity had led to a general feeling that even quite close relations in the human population probably differ sufficiently in their histocompatibility makeup to provide a fully adequate stimulus for a rejection reaction of maximal intensity. Although this is generally true, it has been amply demonstrated in several species that attenuation of the immunological process by, for example, the administration of immunosuppressive drugs, may reveal an entire spectrum of varying histocompatibility differences not previously detectable under the cloak of the maximum intensity reaction which had previously existed. The importance of developing methods for measuring the histocompatibility differences between individuals has thus gained a new and practical urgency in addition to the abiding theoretical importance which has been clear for a longer time.

Since at the present state of affairs much revolves about the question of the details of techniques, this meeting is to be followed by a series of sessions at the laboratory bench. These practical demonstrations will be summarized in the printed version of the proceedings. This later session is due to the zeal and perception of Dr. D. Bernard Amos who has arranged for it to take place at Duke University Medical Center.

Our present meeting has been designed to reveal the cross-section of the different approaches presently being made to the problem of histocompatibility testing. Although full identification and characterization of histocompatibility antigens may be recognized by many to be the ultimate goal, much vital information can be learned by other methods. This conference represents an attempt to gather a small but representative group of leaders in this active field to permit our mutual education and, through the resultant publication, of others not here present. I should like to express our common gratitude to our hosts here at the Academy, to the skillful assistance of Dr. Coyl and Mrs. Manning who have done their usual splendid job of organization, and to my fellow committee members for their enlightened participation.

Since this is to be a 1-day conference, the Committee considered that it would be helpful to gather everyone together for a preliminary evening session at which time our topic could be introduced. Dr. Leslie Brent has contributed very substantially to the field which gathers us all here. So it gives me particular pleasure to turn the floor over to Dr. Brent to set the stage for our considerations.

Some Remarks on the Present State of the Problem of Tissue Typing

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The transplantation of allogeneic kidneys in man is now widely practiced, and at the recent conference on Human Kidney Transplants a valiant attempt was made to evaluate the results obtained at some of the chief clinical centers (Murray, 1964). Enthusiasts as well as skeptics have no doubt derived some support from the data presented by Murray, for, although there have been some notable successes, the long-term survival of patients—and I am thinking in years rather than months—is still depressingly low. There are at least four ways in which the situation is open to improvement: (1) the provision of a test which makes it possible to choose donors whose tissues are at least partially acceptable to the recipients, i.e., a typing test; (2) the development of methods which, with the aid of immunosuppressive agents, induce specific tolerance of the graft; (3) the discovery of less toxic immunosuppressive drugs, of drugs with known antidotes, or of new methods causing impairment of immunological function; and (4) improvements in the management of patients and in the administration of drugs.

To my mind it is the first of these approaches (in conjunction with the second) which holds the key to future progress in clinical tissue transplantation. This view is sometimes challenged because of (1) the belief that nearest relatives must be the closest antigenically; (2) the fact that, where living donors are concerned, only a few close relatives are generally available; (3) the virtual certainty that even an effective typing system would fail to find donors whose tissues are *wholly* acceptable to the patients; and (4) the recent demonstration that, with the aid of the usual immunosuppressive agents, even primate kidneys may function in man for some weeks or even months (Reemtsma *et al.*, 1964). At the risk of preaching to the converted I wish to consider why none of these objections weakens the case in favor of a typing test, however crude it may be to begin with.

It is, of course, perfectly true that for any given recipient the chance of finding a donor whose tissues will be permanently accepted is almost negligible. Good (1952) calculated that the chance of compatibility in randomly chosen donor-recipient pairs is about 1 in 300, but as Newth (1961) has pointed out, this figure is likely to be over-optimistic because Good assumed that the presence or absence of any one antigen is independent of the presence of all others. Newth went over the same ground again and produced a table based on a series of assumptions concerning the number of transplantation antigens and allelic loci in man. I have extracted from his table data on the chances of compatibility (per thousand) between random pairs, between parents and children, and between siblings, for (1) the rather optimistic assumption of 20 antigens and 5 loci, and (2) the perhaps unduly pessimistic assumption of 50 antigens and 20 loci:

No. of antigens	*No. of loci*	*No. of alleles at each locus*	*Random pairs*	*Parents & children*	*Siblings*
20	5	8, 4, 3, 3, 2	0.795	23	57
50	20	8, 4, 3, 3, and 16 × 2	0.00069	0.3067	1.406

Because we do not know how many antigens and genetic loci are involved, or to what extent man follows the mouse in having "weak" as well as "strong" loci, we cannot tell just how realistic these sets of figures are. But, as it seems highly probable that the number of antigens exceeds 20 (Longmire *et al.*, 1947), the truth may lie somewhere between them. At any rate, they lend support to the thesis that it would be entirely unreasonable to expect any typing test, no matter how refined, to solve all our problems in one clean stroke. What, then, are the demands we may make of such a test?

(1) It should exclude from any given panel of potential donors, whether small or large, those whose organs would elicit a particularly violent response, i.e., it should act as an exclusion test; (2) it should pinpoint the individual whose tissues would be tolerated for the longest possible time, i.e., it should act as a selection test; (3) it should not lead to sensitization of the future recipient; (4) it should be quick to execute and have a high degree of discrimination; (5) it must not be subject to disqualification because the patient is uremic or otherwise debilitated; and (6) it should be applicable to cadaver donors (though it is probably true that the more effective the test, the greater will be ethical justification for the use of living donors). Ideally, the test should mimic the blood-group compatibility test in establishing in the human population classes of "more or less compatible" individuals, rather than act as an *ad hoc* arrangement to meet a particular situation. In fact, any test which is not based on an *in vitro* analysis and which does not lead to a systematic, absolute classification should be regarded as no more than a temporary substitute.

What do I mean by "more or less compatible" and what bearing might such selection have on the long-term survival of, let us say, a human kidney allograft? Here we must turn to the mouse for guidance, for we know more about the genetics and immunology of histoincompatibility of the mouse than of any other species. There are loci controlling the expression of "strong" and of "weak" transplantation antigens, so that, depending on the loci at which donor and recipient differ, a skin graft may be rejected quickly or slowly (Counce *et al.*, 1956). The difference in survival time may be only a matter of a few days, but there is plenty of evidence indicating that it may be of vital importance in any attempt to subdue the immunological response. Here are a few examples:

(1) When inducing tolerance in newborn mice, 5×10^6 allogeneic spleen cells per gram of body weight are required for a strong combination, but a mere 0.15×10^6 for a weak one (Brent and Gowland, 1962). (2) Using a *very* weak combination (coisogeneic strains with normal survival times of 19 to 21 days), tolerance can be induced in adult mice with a single injection of 200×10^6 cells (McKhann, 1962); on the other hand, without the assistance of immunosuppressive agents prolongation of skin-graft survival can be achieved—if at all—in fully adult mice of a *strong* combination only by the injection of very large numbers of cells spread out over a long period of time (Shapiro *et al.*, 1961); in fact, in experiments of very similar design Gowland and I (unpublished) have completely failed to induce tolerance in fully adult, as opposed to adolescent, mice. (3) Using intravenously administered cell-free tissue extracts and immunosuppressive therapy such as X-rays or a-methopterin, long-term prolongation of skin-graft survival is brought about much more readily in a weak combination (Medawar, 1963).

Because the difference in normal survival times between weak and strong combinations is only a matter of days, these experimental results should encourage us to seize on any typing system offering a comparable degree of discrimination between potential donors. The fact that in practice living donors tend to be close relatives does not, of course, invalidate this approach; in fact, both theoretical estimates and clinical experience indicate that it would greatly increase the chances of finding a partially compatible donor.

What, then, does one make of the recent reports that primate kidneys have functioned for surprisingly long periods in man (Reemtsma *et al.*, 1964)? If even species differences do not constitute an insurmountable barrier, are we not wasting our time trying to distinguish between what have always been regarded as the finer and more subtle individual-specific differences? It seems to me that such a conclusion would be quite untenable. Although the even limited survival of primate kidneys in man is certainly unexpected, it has been brought about with the aid of massive doses of immunosuppressive drugs. One of the advantages of the typing approach is that, by picking out the partially compatible donor, it should make it possible to lower the drug dose and to reduce the mortality directly or indirectly attributable to the patient's treatment. Furthermore, at present we know very little

about the nature of species and individual histocompatibility differences in primates and it could conceivably turn out that the typing techniques worked out for allografting in man also have some significance for xenogeneic grafts.

As far as I know, six typing methods have so far been offered as possible contenders: blood typing, platelet typing, leukocyte typing, third-party skin grafting, *in vitro* stimulation of lymphocytes, and the normal lymphocyte transfer test. In addition, I wonder whether *in vitro* macrophage inhibition (David, 1964) might not lend itself as an indicator of immunological reactivity following organ transplantation, in that it might reveal an impending rejection crisis more quickly than the currently used physiological methods. It would be foolhardy of me to try to assess the relative merits of these different tests; that is the purpose of our conference. I wish to add only that none of them need exclude the others, and that until a wholly satisfactory typing test has been devised—and it seems to me that this will almost certainly take the form of serological discrimination of one sort or another—it may well be that at least some of them ought to be used in conjunction.

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Platelet and Leukocyte Isoantigens

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1. Serology

a. *Early work.* A number of investigators in the period 1954-1959 concluded that platelets and leukocytes possessed many of the antigens present on erythrocytes, e.g., A, B, (Dausset, 1954; Coombs and Bedford, 1955; Gurevitch and Nelken, 1954; Ashhurst, Bedford, and Coombs, 1956; Ducos, Broussy, and Ruffie, 1959) C, D, E, (Ducos, Broussy, and Ruffie, 1959; Dausset, Colombani, and Evelin, 1958; Moulinier and Servantie, 1958) M, N, Tj^a, (Ashhurst, Bedford, and Coombs, 1956; Ducos, Broussy, and Ruffie, 1959) and others, (Ashhurst, Bedford, and Coombs, 1956; Ducos, Broussy, and Ruffie, 1959) but there were some reports to the contrary, e.g., C, D, E, (Ashhurst, Bedford, and Coombs, 1956; Gurevitch and Nelken, 1957). More recent reports strongly suggest that the presence of contaminating erythrocyte stroma (Swisher, 1958; Barnes *et al.*, 1963; Bakemeier and Swisher, 1957) or soluble A or B substances adsorbed on platelets (Lewis, Draude, and Kuhns, 1960) may have caused false-positive reactions with the agglutination and adsorption techniques that were used. The work of Bakemeier and Swisher (1957), in particular, points out the difficulties of interpreting serologic tests that may result in nonspecific "mixed agglutination" of leukocytes or platelets with intact or lysed erythrocytes. In view of conflicting reports and the fact that techniques used were susceptible to false-positive reactions, it seems likely that erythrocyte isoantigens either are not present on leukocytes and platelets or are present only in relatively insignificant amounts.

In 1957 Moulinier, using an exceptional antibody from a mother whose infant had neonatal purpura, was able to obtain positive antiglobulin consumption tests with 22 percent of 82 platelet suspensions and named the reactive antigen $DUZO^a$. Dausset (1958), on the basis of finding that seven different antisera from transfused individuals gave a similar pattern of agglutination with 20 leukocyte suspensions, named the antigen that appeared to be present on 12 of the leukocytes "Mac."

b. *Recent work.* In the past few years, several investigators have taken advantage of the simplified agglutination technique of Dausset and Malinvaud (1954) to identify platelet isoantigens with strong platelet agglutinins that occasionally arise after transfusion. The technique is insensitive but gives unequivocally reproducible results with the rare agglutinins that are unearthed by screening hundreds of sera from transfused individuals. With this technique, van Loghem *et al.* (1959) and van der Weerdt *et al.* (1963) found two antibodies that recognized allelic antigens they called Zw^a and Zw^b; and van der Weerdt *et al.* (1962) and Dausset and Berg (1963), using the same technique, found an agglutinin against a third platelet antigen that was named Ko. Van Rood and van Leeuwen (1963), in a formidable undertaking with a leukoagglutinin procedure that was subject to several technical snares, selected (by statistical analysis with a computer) from 66 reactive maternal sera those that appeared to recognize allelic antigens and designated the alleles 4^a and 4^b.

With complement-fixation techniques and sera from two patients who had developed thrombocytopenia after blood transfusion, we identified a platelet isoantigen that was called Pl^{A1} (Shulman *et al.*, 1961). An attempt was made to follow the nomenclature recommendations of Ford (1955). Anti-Pl^{A1} proved to be identical with anti-Zw^a of van Loghem when serum samples were exchanged between laboratories. The antigen Pl^{A1} is therefore the same as Zw, or Zw^a as it was called when the allelic antigen was recognized (van der Weerdt *et al.*, 1963). By precedence, the antigen should be referred to as Zw^a. However, with the prospect

that many new antigens will be defined in the near future, some system of nomenclature should be applied. We will therefore use the designation Pl^{A1}, and have used the same system in naming a number of other platelet and leukocyte isoantigens. With antibodies from mothers whose infants had neonatal purpura and with sera from transfused patients, we subsequently identified the antigens $PlGrLy^{B1}$ (Shulman, Aster, Pearson, and Hiller, 1962; Shulman, Marder, Aledort, and Hiller, 1962; Pearson *et al.*, 1964), and $PlGrLy^{C1}$ (Shulman, Marder, Aledort, and Hiller, 1962; Pearson *et al.*, 1964), which are shared by platelets, granulocytes, and lymphocytes, the antigen Ly^{D1} (Shulman, Marder, Aledort, and Hiller, 1962), which is present only on lymphocytes, and recently the allelic platelet antigens Pl^{B2} and Pl^{B3} (Shulman, Marder, Hiller, and Collier, 1964).

Tables 1 and 2 summarize the data available at present on specific platelet and leukocyte isoantigens and their antibodies. Shulman, Marder, Hiller, and Collier (1964) review in detail the serology of monospecific isoanti-

TABLE 1

Serologic Characteristics of Monospecific Antibodies Against Platelet and Leukocyte Antigens*

Anti-	*Aggl.*	*Anti-glob. Consumption* γ	*Anti-glob. Consumption* C′	*C′ Fix.*	*Inhib. Cl. Retr.*	*Inhib. C′ Fix.*	*Cell Specificity*
(1) Pl^{A1} or Zw^a	+	+	o	+	+		Platelets
Incomplete form	o	±	o	o	o	+	
(2) Pl^{A2} or Zw^b	+	o	o	o	o		Platelets
(3) $PlGrLy^{B1}$	o	o	o	+	±		Platelets, Granulocytes, Lymphocytes
Incomplete form	o	o	o	o	o	+	
(4) $PlGrLy^{C1}$	o	o	o	+	o		Platelets, Granulocytes, Lymphocytes
Incomplete form	o	o	o	o	o	+	
(5) Ly^{D1}	o	o	o	+			Lymphocytes
(6) Pl^{B1}	o	±	o	+	±		Platelets
(7) Pl^{B3} (Probable)	o	o	o	+	o		Platelets
(8) Ko^a	+	o	o	o	o		Platelets
(9) 4^a	+			o			Granulocytes, Platelets**
(10) 4^b	+			o			Lymphocytes**
(11) $DUZO^a$		+					Platelets
(12) Mac	+						Leukocytes

* This table is modified from Shulman, Marder, Hiller, and Collier (1964).
** By adsorption only; reactions of these cells not tested.
References: (1) van Loghem *et al.*, 1959; van der Weerdt *et al.*, 1963; Shulman *et al.*, 1961; Shulman, Aster, Pearson, and Hiller, 1962; Shulman, Marder, Aledort, and Hiller, 1962; Pearson *et al.*, 1964; Shulman, Marder, Hiller, and Collier, 1964; Shulman, 1963; Shulman, 1964; (2) van der Weerdt *et al.*, 1963; Shulman, Marder, Hiller, and Collier, 1964; (3) Shulman, Aster, Pearson, and Hiller, 1962; Shulman, Marder, Aledort, and Hiller, 1962; Pearson *et al.*, 1964; Shulman, Marder, Hiller, and Collier, 1964; (4) Shulman, Marder, Aledort, and Hiller, 1962; Pearson *et al.*, 1964; Shulman, Marder, Hiller, and Collier, 1964; Shulman, Marder, Aledort, and Hiller, 1964; (5) Shulman, Marder, Aledort, and Hiller, 1962; Shulman, Marder, Aledort, and Hiller, 1964; (6) Shulman, Marder, Hiller, and Collier, 1964; (7) Shulman, Marder, Hiller, and Collier, 1964; (8) van der Weerdt *et al.*, 1962; Dausset and Berg, 1963; (9) van Rood and van Leeuwen, 1963; (10) van Rood and van Leeuwen, 1963; (11) Moulinier, 1957; (12) Dausset, 1958.

TABLE 2

Source of Antibodies, Percent Reactors, and Genotype Frequencies*

Anti-	Source		Panel		Approximate Genotype Frequency		
	Post-transfusion	Maternal	Total	% Positive	Approx. Gene Frequency	Homozygous	Heterozygous
(1) Pl^{A1} (Zw^a)	4	17	452 287**	97 97.6	.83	.69	.28
(2) Pl^{A2} (Zw^b)	1		435 75†	26 27	.17	.03	.28
(3) $PlGrLy^{B1}$	5	8	888	46	.26	.07	.39
(4) $PlGrLy^{C1}$	1	2	252	30	.17	.03	.28
(5) Ly^{D1}	2	1	113	36	.20	.04	.32
(6) Pl^{E1}	1		945	>99.9	.975	.95	.049
(7) Pl^{E2}		1	147	5	.025	.0006	.049
(8) Ko^a	4		435 75††	17 12	.08	.005	.145
(9) 4^a		5	347	63	.38	.15	.47
(10) 4^b		7	347	86	.62	.38	.47
(11) $DUZO^a$		1	82	22	.12	.014	.21
(12) Mac	5		20	60	.37	.14	.47

* This table is reproduced from Shulman, Marder, Hiller, and Collier, 1964.
** This panel was tested by van Loghem *et al.* (1959) by agglutination.
† This panel was tested by us with serum sent by van der Weerdt *et al.* (1963).
†† This panel was tested by us with serum sent by van der Weerdt *et al.* (1962) and by Dausset and Berg (1963).

bodies against platelet and leukocyte isoantigens, with emphasis on principles that can be used to define new ones, present examples of the use of specific isoantibodies in pathophysiologic studies, and relate experimental studies to problems of clinical medicine.

c. *Critique of techniques.* Four general techniques have been used to characterize platelet and leukocyte isoantigens: agglutination, antiglobulin consumption, inhibition of clot retraction, and complement fixation. Each has its own advantages and disadvantages; there is no single test that is reliable for measuring all antibodies. The advantages of the platelet agglutination test of Dausset and Malinvaud (1954) are its simplicity and clarity, but the test is infrequently applicable because of the rarity of strong agglutinins, and it is inconvenient for use with large panels because fresh cells are required. Leukocyte agglutination (van Rood and van Leeuwen, 1963) is a sensitive test with some antibodies, but does not detect others, is subject to many technical pitfalls, is particularly difficult to use for selecting antibodies against single antigens, and requires fresh cells. The antiglobulin consumption test (Moulinier, 1957; Steffen, 1960) requires large amounts of antibody and fresh cells, is cumbersome to perform with more than a few cell samples, and is subject to great variation in results with minor changes in procedure (van de Wiel, van de Wiel-Dorfmeyer, and van Loghem, 1961; Engelfriet and van Loghem, 1961). Antiglobulin consumption is, however, the only test available for detecting those incomplete antibodies that are not measurable by inhibition of complement fixation (Shulman, Marder, Hiller, and Collier, 1964). The very simplest technique, inhibition of clot retraction, may be useful only on rare occasions to identify an isoantibody and phenotype cells (Shulman *et al.*, 1961; Shulman, Marder, Hiller, and Collier, 1964). Complement fixation is a sensitive technique for measuring both platelet and leukocyte isoantibodies (Shulman, Marder, Hiller, and Collier, 1964), can be used with stored or shipped cells, detects some blocking antibodies not measurable by other methods, can sometimes be used to determine gene dosage effects (Shulman *et al.*, 1961; Shulman, Marder, Hiller, and

Collier, 1964), permits quantitative measurements of antigens and antibodies, and gives clear-cut results easily with large panels of cells when used for qualitative measurements (Shulman, Marder, Hiller, and Collier, 1964).

Other techniques that have been used in an attempt to detect isoantibodies and heteroantibodies, but have not been applicable in defining antigenic specificity of isoantibodies, are: agglutination techniques involving prolonged incubation at 5°C (Harrington, Minnich, and Arimura, 1956; Shulman, 1958) fluorescent antibody methods (Silber *et al.*, 1960; Vazquez and Lewis, 1960), the mixed antiglobulin reaction (Chambers, Coombs, and Gurner, 1959), the tanned erythrocyte hemagglutination test (Kissmeyer-Nielsen, 1953), and techniques involving labeling of antisera or antiglobulin with I^{131} (Anderson and Walford, 1960). Not all of these tests have been given a thorough trial with each type of isoantibody, but the indications are that these techniques either are useless or offer no improvement over simpler techniques that have been used to define monospecific isoantibodies. Approaches involving various measurements of cytotoxicity effects (Terasaki and Rich, 1964) or mitogenic effects in tissue culture (Gräsbeck, Nordman, and de la Chapelle, 1963), have not yet been explored with human isoantibodies.

2. Interspecies Relationships

Table 3 shows the heterologous reactions of human antibodies against platelet and leukocyte isoantigens. Those isoantigens that were products of genes occurring frequently in the human population (see Table 2) were found on cells of all other primates tested, whereas antigens that were products of human genes that occur relatively infrequently were absent on cells of other primates. No human platelet or leukocyte isoantibodies have been found to react with guinea pig or rat platelets (Shulman, Marder, Hiller, and Collier, 1964), and anti-Pl^{A1} is the only human platelet or leukocyte isoantibody that has reacted with cells of dogs and rabbits. It is interesting that, with animal platelets, anti-Pl^{A1} reacted only to fix complement and did not cause agglutination or inhibition of clot retraction, as it did with human platelets. The species specificity of anti-Pl^{A1} reaction is sufficiently distinctive to permit tentative identification of this antibody in other laboratories without an exchange of sera.

All of our attempts to immunize rabbits, guinea pigs, rats and dogs against human platelet and leukocyte isoantigens have been unsuccessful (Shulman, Marder, Hiller, and Collier, 1964). These animals developed

TABLE 3

Heterologous Reactions of Human Platelet and Leukocyte Isoantibodies*

Platelets From	*Antigenic Specificity of Human Antibody*									
	Pl^{A1}		$PlGrLy^{B1}$		$PlGrLy^{C1}$		Ly^{D1}		Pl^{E1}	
	No. Tested	*% Pos.*	*No. Tested*	*% Pos.*	*No. Tested*	*% Pos.*	*No. Tested*	*% Pos.*	*No. Tested*	*% Pos.*
Man	452	97	888	46	252	30	113	36	945	>99.9
Chimpanzee	54	100	32	0	37	0			56	100
Orangutan	35	100	21	0	22	0			36	100
Baboon	26	100	23	0	23	0			32	100
Rhesus Monkey	12	100	9	0	10	0	12	0	9	100
Gibbon	10	100							10	100
Dog	21	100	20	0	20	0	6	0	12	0
Rabbit	35	100	20	0	20	0	6	0	12	0
Guinea Pig	12	0	10	0	10	0	6	0	6	0
Rat	16	0	10	0	10	0			6	0

* This table is reproduced from Shulman, Marder, Hiller, and Collier, 1964.

high-titered anti-human platelet and leukocyte antibodies, but the antibodies did not discriminate isoantigens. Any one human platelet or leukocyte preparation adsorbed out heterologous antibodies for all other cell preparations. Attempts to stimulate anti-Pl^{A1} by immunizing rats that are Pl^{A1}-negative with Pl^{A1}-positive platelets from a more closely related species, rabbit, did not produce antiserum that would react with Pl^{A1}-positive human platelets (Shulman *et al.*, 1961). Possibilities of developing type-specific antisera against human platelet and leukocyte isoantigens in lower animals have not been fully exploited, but the likelihood of success seems slight.

Cells from nonhuman primates could sometimes be used as effectively as human cells to dissect mixtures of isoantibodies. Table 4 shows the reactions of a serum from a repeatedly transfused patient, containing a mixture of antibodies that fixed complement with all human and chimpanzee platelets tested, and with some baboon, orangutan, and gibbon platelets, but not with Rhesus monkey platelets. An eluate made from this serum with chimpanzee platelets contained an antibody that reacted with some chimpanzee and human platelets and not with others; and an eluate prepared from the serum with human cells that had reacted with the chimpanzee eluate contained an antibody that reacted concordantly with all of the same cells. The chimpanzee and human eluate therefore appeared to contain the same monospecific antibody. By using cells from a species that has some, but not all, human antigens, the chances of obtaining a monospecific eluate from mixtures of isoantibodies may be better than with human cells. Baboon cells that had reacted with the whole serum did not react with the chimpanzee or human cell eluates (Table 4). Had they been sufficiently available, baboon cells would have been the cells of choice to use for preparing an eluate in an attempt to identify a second monospecific isoantibody. This type of analysis need not be done near the source of nonhuman primates, for complement-fixation procedures permit using shipped and stored cells.

Opportunities to use human isoantibodies to identify antigens of other primates and to use cells of various primates to identify human antigens are numerous, for we have found that five of six sera with mixtures of human isoantibodies cross-reacted with platelets, leukocytes, or both, from members of one or more primate species. Moreover, it was found that chimpanzees immunized with human whole-blood or buffy-coat suspensions developed antibodies that reacted with platelets, leukocytes, or both, of some, but not all, human beings. The complement-fixing antibodies developed in chimpanzees had all the characteristics of human nonagglutinating isoantibodies that are directed against specific human isoantigens.

A detailed report of the work involving nonhuman primates, done in association with Dr. Moor-Jankowski, will appear shortly.

TABLE 4

Dissection of a Human Antiserum with Cells from other Primates*

Platelets From	*Serum from Patient K. I.*					
	Whole		*Eluate Chimp.*		*Eluate Human*	
	No. Tested	*No. Pos.*	*No. Tested*	*No. Pos.*	*No. Tested*	*No. Pos.*
Man	80	80	46	31	46	31
Chimpanzee	38	38	12	3	12	3
Baboon	28	11	6	0	6	0
Orangutan	20	13				
Gibbon	10	9				
Rhesus Monkey	12	0				

* This table is reproduced from Shulman, Marder, Hiller, and Collier, 1964.

3. Isoantigens and Histocompatibility

The possible role of blood-group isoantigens in homograft immunity and the relative importance of classical serologic reactions and delayed cellular hypersensitivity in graft rejection have been discussed in a number of recent symposia (Albert and Medawar, 1959; Wolstenholme and Cameron, 1962; Johnson, 1962) and reviews (Brent, 1958; Medawar, 1958; Amos, 1962). It has been concluded that leukocytes and, in some species, possibly platelets and erythrocytes share skin antigens responsible for homograft immunity because (1) sensitization to blood leukocytes by the intradermal, and to a lesser extent by the intravenous, route, in certain animals (Medawar, 1946), and by the intradermal route in man (Friedman *et al.*, 1961), results in immune skin-graft rejection; (2) skin grafts sometimes induce antibodies against leukocytes and red cells detectable by serologic tests, particularly in mice (Amos, 1962; Gorer, 1956), or against platelets detectable by shortened platelet survival in rabbits (Ebbe, Baldini, and Dameshek, 1962); and (3) sensitization to grafted skin produces delayed cutaneous hypersensitivity that can be evoked by intradermal injections of leukocytes in guinea pigs (Brent, Brown, and Medawar, 1958) and man (Merrill *et al.*, 1961). Although it is evident that leukocytes elicit transplantation immunity against other tissues, it is not yet clear whether the responsible antigens are the same as those that stimulate circulating antibodies or cause cutaneous hypersensitivity, and, if so, whether they are typical blood-group isoantigens.

It seems likely, from various observations that have been made in man, that homograft rejection will not usually be associated with circulating antibodies against tissues or blood cells that are measurable directly by serologic tests available at present. However, relationships between blood-group isoantigens and histocompatibility antigens can be evaluated with monospecific isoantibodies and current serologic techniques in the following types of analyses.

a. *Graft rejection and phenotypic incompatibility.* On occasions when skin, kidney, bone marrow, or other tissue is transplanted in man, determinations of donor and recipient phenotypes with all available monospecific antisera against platelet and leukocyte isoantigens may permit identifying mismatches of significance with respect to graft rejection. Isoantigens not shared by blood cells and immunizing tissues, as determined by serologic adsorption procedures (van Rood and van Leeuwen, 1963; Shulman, Marder, Hiller, and Collier, 1964), could be eliminated as transplantation antigens.

b. *Blood-cell survival and homograft immunity.* Individuals who have rejected grafts, but have no serologically active isoantibodies against platelets or leukocytes may nevertheless have developed antibodies detectable by their ability to shorten the *in vivo* survival of platelets (Ebbe, Baldini, and Dameshek, 1962) or leukocytes. By using cells that are antigenically well defined in survival studies, it may be possible to associate specific isoantigens with homograft immunity.

c. *Cutaneous hypersensitivity and specific isoantigens.* If transplantation antigens are similar to the usual blood-group polysaccharide isoantigens, one would not expect them to cause cutaneous hypersensitivity, for polysaccharides in general have not elicited this type of reaction (Waksman, 1960) and leukocyte (Merrill *et al.*, 1961), platelet (Shulman *et al.*, 1961) and erythrocyte isoantigens (Jandl and Tomlinson, 1958) in particular have not caused delayed cutaneous reactions in individuals sensitized by the intravenous route. However, relatively few isoantigens have been tested in this way, and it is conceivable that the very blood-group isoantigens responsible for homograft immunity will elicit cutaneous hypersensitivity. Skin tests performed with antigenically well defined leukocytes on individuals known to be sensitized by transfusion or pregnancy, as well as by grafts, should help to resolve this problem.

d. *Heterotransplantation and isoantigens.* In those instances in which heterotransplants in man are performed therapeutically [e.g., dog or monkey parathyroid to man (Stone, Owings, and Gey, 1934) and ape kidney to man (DeWitt *et al.*, 1964)] evaluation of heterotransplantation antigens on blood cells may be sought not only by the usual serologic and skin tests, but also by passive transfer of plasma from immunized recipients to donor animals, for suppression of circulating blood cells is frequently the most sensitive test for antibody (Shulman, Marder, and Weinrach, in press). If certain isoantigens common to human and nonhuman primates (see Tables 3 and 4) prove to be significant in histocompatibility, it may be possible to perform transplantation experiments in animals that will have direct bearing on the problem in man.

e. *Physicochemical properties.* Pure forms of transplantation antigens and leukocyte or platelet blood-group isoantigens are difficult to obtain (Shulman *et al.*, 1961; Herzenberg and Herzenberg, 1961; Davies, 1962); hence, their chemical characteristics are not established. However, stability properties of transplantation antigens (Medawar, 1958) and leukocyte isoantigens (Shulman, Marder, Hiller, and Collier, 1964) under different forms of treatment, e.g., pH, temperature, solvents, and enzymes, were consistent with their both being polysaccharides. More recently, analyses on refined transplantation antigens have suggested that they are lipoproteins (Herzenberg and Herzenberg, 1961; Davies, 1962), but materials used in these studies were insufficiently pure to rule out the possibility of their being polysaccharides. As yet it is not possible to draw conclusions concerning similarities or differences between transplantation antigens and isoantigens on the basis of their physicochemical characteristics.

f. *Maternal-fetal histocompatibility.* Since maternal sensitization to fetal platelets and leukocytes most often results in formation of monospecific isoantibodies (Shulman, Marder, Aledort, and Hiller, 1962; Pearson *et al.*, 1964; Shulman, Marder, Hiller, and Collier, 1964), the significance of certain incompatibilities in transplantation immunity may possibly be derived from graft exchanges between immunized mother and offspring in the manner used by Peer (1958) and others to evaluate apparent maternally induced tolerance in children. The effect of specific isoimmunization on the mother's acceptance of her offspring's skin would be difficult to predict on the basis of observations made in animals, for the ability to reject grafts was found to be weakened by prior intravenous injection of homologous leukocytes or epidermal cells in rabbits (Billingham and Sparrow, 1955), but strengthened by similar injections in mice and rats (Billingham, Brent, and Mitchison, 1957; Steinmuller and Weiner, 1963).

Approximation of placental trophoblastic tissue to maternal circulation would seem to afford an opportunity for immunization, but homograft reactions between mother and fetus do not occur. This is apparently owing to a deficiency of transplantation antigens in the trophoblast in those species that have been studied (Hašek *et al.*, 1962; Simmons and Russell, 1962). If human trophoblastic tissue proves to lack transplantation antigens also, then leukocyte and platelet isoantigens that are shared by placental tissue may be excluded as transplantation antigens. For example, the 4^a and 4^b leukocyte antigens of van Rood could be excluded, for they are present in the placenta (van Rood and van Leeuwen, 1963).

g. *Choriocarcinoma.* This malignancy might be expected to elicit isoantibodies, for it is genetically dissimilar to the host. However, sera of patients with advanced choriocarcinoma studied by Schmidt and Hertz (1961) did not contain detectable antibodies against erythrocyte blood-group antigens of their husbands; more recently, using 33 of the same sera, we did not find $C^{\prime}$-fixing antibodies against platelet and leukocyte isoantigens that could be attributed to sensitization by trophoblastic tissue (Shulman, Marder, Hiller, and Collier, 1964). Moreover, trials of treating choriocarcinoma in women by immunizing them with their husband's blood have not been successful (Doniach, Crookston, and Cope, 1958; Hackett and Beech, 1961). These various observations suggest that trophoblastic tissue is deficient in both histocompatibility antigens and isoantigens. However, if rejection of choriocarcinoma in some instances is associated with development of antibodies against a particular isoantigen, or if particular isoantibodies stimulated by choriocarcinoma do not cause tumor rejection, the information may provide clues to principles of tissue rejection equally applicable to problems of immunologic tumor therapy and homotransplantation.

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An *in Vitro* Expression of Homograft Sensitivity*

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The technique of peritoneal cell migration from capillary tubes has been applied to the study of delayed allergy in guinea pigs (George and Vaughan, 1962; David *et al.*, 1964). Using this technique it was observed that the migration of peritoneal cells from guinea pigs with delayed allergy (e.g., to tuberculin and protein antigens) is inhibited only in the presence of the specific antigen. In contrast, the migration of peritoneal cells from guinea pigs producing circulating antibody was not affected by the specific antigen and normal cells incubated with antibody were also not inhibited by antigen. We have extended this technique of cell migration to the study of the homograft reaction in inbred strains of mice. The inhibition of cell migration was used as the endpoint in this system. In this report evidence will be presented to indicate that specific inhibition of migration occurs when cells from mice expressing homograft sensitivity are mixed with cells from the strains used to sensitize.

Material and Methods

The mice used in this study (A/Jax, CBA, and C57BL/6) were obtained from the Jackson Memorial Laboratory, Bar Harbor, Maine. These mice were 3 months old at the time of the experiment. A/Jax mice donated the sensitizing skin grafts, and CBA mice served as recipients.

A single skin homograft was applied to the lateral thoracic region. These grafts were suprapannicular 11 mm in diameter. They were covered with nonadhering dressing, then wrapped with latex bandage and adhesive tape.

Peritoneal cells were harvested in cold Hank's solution 3 to 4 days following the intraperitoneal injection of 3 to 4 ml of light mineral oil. In the case of sensitive mice, mineral oil was injected after the rejection of the sensitizing skin grafts. The cells were suspended in Eagle's culture medium containing 15 percent normal guinea pig serum, packed in capillary tubes and placed in tissue-culture chambers as described by David *et al.* (1964). When cells from two different strains were mixed, equal proportions were used and the cells were mixed thoroughly and then loaded into the capillary tubes.

Assay System

The tissue-culture chambers are incubated for 24 hr at 37°C. The surface area of migration is measured by planimetry, and the results calculated according to the following formula:

$$\% \text{ migration of cell mixture} = \frac{\text{average migration of mixture of cells}}{\text{average migration of normal \& sensitive cells separately}} \times 100$$

Results

The results are presented in Table 1. It can be seen that inhibition of migration occurred in all the experiments in which cells from sensitive CBA mice were mixed with cells from A/Jax mice (the sensitizing strain). This inhibition is specific, since cells from sensitive CBA mice migrated normally when mixed with cells from C57BL/6, unrelated

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TABLE 1

Migration of Peritoneal Cells from Normal and Sensitive CBA Mice in the Presence of Cells from the Sensitizing Strain A/Jax and from a Nonsensitizing Strain

	No. of animals (pooled)	*Migration, %*
CBA sensitive to A/Jax + A/Jax	15	55
	15	70
	15	56
	20	55
Normal CBA + A/Jax	15	106
	16	97
	15	100
CBA sensitive to A/Jax + C57BL/6	20	101

to the sensitizing strain A/Jax. Furthermore, mixture of cells from normal CBA and A/Jax mice migrated well without evidence of inhibition.

Discussion

Evidence is presented in this study that inhibition of cell migration occurs only when peritoneal cells from mice with induced homograft sensitivity are mixed with peritoneal cells from the specific strain that had donated the sensitizing skin grafts. Since both populations of cells in the mixture are viable and capable of migration, the inhibition observed could be due to an effect on either of the populations or to a composite effect on both.

Since cells from the donor animals are possessed of homograft antigens, the inhibition of migration could result from an interaction of antigens (residing in or on the surface of donor cells) with sensitive lymphoid cells. This is similar to the delayed-allergy model in guinea pigs, except in that system soluble antigens interact with sensitized cells in the medium instead of the viable cell-to-cell interaction employed in this study.

It may be that the inhibition of migration observed in this study is related to the effect (i.e., sickness or death) on target cells by sensitive lymphoid cells. Thus, peritoneal cells from sensitive CBA mice may exert a damaging effect on cells from the donor strain A/Jax, interfering with their migration. Rosenau (1963) showed that spleen cells from sensitive mice are capable of destroying *in vitro* cells of the type that had induced the sensitivity. A similar observation was made by Old *et al.* (1963), using an *in vitro* system to demonstrate destruction of ascites sarcoma cells by peritoneal macrophages from mice immunized against these tumors. However, when the peritoneal macrophages were washed, this capacity was abolished. This latter finding is in contrast to our system, where peritoneal cells are washed prior to their use.

It is conceivable, however, that the inhibition of migration may be due to donor and sensitive cells affecting each other simultaneously.

It should be noted that under the conditions of the present experiment (24 hr observation) mixtures of peritoneal cells from two normal but genetically different strains of mice migrated well together without affecting each other. This is of interest, since the cells in the mixture are in close contact and both populations are immunologically competent.

This technique offers particular potential for assay and characterization of homograft antigens and may prove to be a useful adjunct for studies on homograft sensitivity.

Summary

The technique of peritoneal cell migration in tissue-culture chambers has been applied to the study of homograft reactions *in vitro*. The migration of cells from inbred mice with homograft sensitivity is inhibited when they are exposed to cells from the specific sensitizing strain.

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Defined Leukocyte Antigenic Groups in Man

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Introduction

The intradermal injection of leukocytes will induce homograft sensitivity in rabbits, as has been shown by Medawar (1946). In men, similar findings have been reported by Friedman *et al.* (1961) and Rapaport, Lawrence, and Converse (1964). The implication of these observations is that some of the isoantigens of the leukocyte will be identical with the transplantation antigens. For that reason, information concerning the leukocyte isoantigens or groups will be relevant to the problem of tissue histocompatibility testing.

The existence of leukocyte groups has been demonstrated by four separate sets of observations. In the first place, leukocyte agglutinins formed after blood transfusions agglutinated leukocytes from a varying number of random donors, but did not agglutinate the leukocytes of the patient who had formed the agglutinin (Dausset, 1954). Dausset (1958), studying the agglutination pattern of a group of 27 sera with a panel consisting of 20 leukocyte samples, noticed that 7 of the 27 sera showed more or less the same agglutination pattern. From the observation that the leukocytes from these patients were not agglutinated by their own serum or by the six other sera from this group, Dausset concluded that they lacked the same antigen, which he designated "Mac." Further proof of the existence of the antigen Mac was furnished by the agglutination pattern of leukocyte agglutinins formed after repeated injections of blood from three donors into six patients. The antigen Mac could be demonstrated in about 60 percent of the leukocyte samples from randomly selected donors.

In the same paper, Dausset reported a second observation which provides additional evidence that leukocyte groups do exist. Monozygotic twins proved to have identical agglutination patterns of their leukocytes, while the agglutination pattern of the leukocytes of dizygotic twins was not identical. Lalezari and Spaet (1959) reported similar findings.

A third argument was found by Payne and co-workers (1958, 1961) and by van Rood *et al.* (1959, 1962) in family studies, which showed that leukocyte isoantigens could be inherited.

Finally, the formation of leukocyte agglutinins during pregnancy was a strong argument in favor of the existence of leukocyte groups (Payne and Rolfs, 1958; van Rood, van Leeuwen, and Eernisse, 1959).

From the results of cross-absorption experiments with two sera with leukocyte agglutinins formed during pregnancy, van Rood *et al.* (1959) postulated the existence of two other leukocyte groups, groups Two and Three.

In 1961 a method was described by which it is possible to select two types of sera with leukocyte agglutinins (van Rood *et al.*, 1962):

(1) sera which recognize the same antigens, and

(2) sera which recognize antigens which are the expression of allelic genes.

This method has been helpful in the delineation of three independent leukocyte groups, each with at least two allelic genes (van Leeuwen *et al.*, 1964; van Rood and van Leeuwen, 1963). Before describing these leukocyte groups and their characteristics, it seems appropriate to discuss the difficulties which are encountered when these studies are carried out and to underline the limitations of the system.

The method can be divided into three stages:

(I) selection of the sera,
(II) cross-absorption studies, and
(III) determination of the genotypes.

I. *The selection of the sera.* The following properties of the sera with leukocyte agglu-

tinins will limit their usefulness for leukocyte typing:

(1) Titers are generally low (under 1/128).

(2) Reproducibility is poor, especially for the weak reactions; the agglutination test with leukocytes from EDTA blood has a better reproducibility than the leukocyte agglutination test using leukocytes from defibrinated blood (van Rood, 1962).

(3) False negative reactions do occur; this phenomenon was named the agglutination-negative-absorption-positive phenomenon (van Rood, 1962).

(4) More than half of the sera have antibodies against more than one antigen.

To overcome these difficulties the following approach has been used: Sixty sera from pregnant women with leukocyte agglutinins were tested against the leukocytes from 100 randomly selected people. The resulting 6000 reactions were analyzed by electronic computers to answer the following questions:

Which sera recognize the same antigen?

Which sera might recognize alleles?

The computer was able to indicate that the 60 sera could be divided into four unrelated groups:

12 sera recognizing allelic antigens which were later called 4a and 4b,

3 sera recognizing the two antigens of group Five,

13 sera recognizing the antigens of group Six, and

32 sera that could not be placed in any of the preceding groups.

II. *The cross-absorption studies.* When a number of sera with similar specificity are found, it is still necessary to prove that they recognize only one antigen. This can be done by cross-absorption experiments for which 12 to 15 donors are selected. The leukocytes of the majority of them must be agglutinated by the serum under study; the remainder, which are not agglutinated, will serve as negative controls. The leukocytes from 70 ml of blood or more from each donor are isolated and used to absorb 0.5 to 1.0 ml of blood of the serum under study. (For an example, see Table 1.) The absorbed

TABLE 1*

Cross-Absorption Experiment with Serum No. 36

	Agglutination reaction of the leukocytes from donor no.:											
	38	*56*	*55*	*50*	*37*	*31*	*51*	*52*	*11*	*44*	*58*	*49*
Before absorption	+++	+++	++	++	++	+	+	+	+	–	–	–
After absorption with leukocytes from donor no.:												
38	–	–	–	–	–	–	–	–	–	–	–	–
56	–	–	–	–	–	–	–	–	–	–	–	–
55	–	–	–	–	–	–	–	–	–	–	–	–
50	–	–	–	–	–	–	–	–	–	–	–	–
37	–	–	–	–	–	–	–	–	–	–	–	–
31	–	–	–	–	–	–	–	–	–	–	–	–
51	–	–	–	–	–	–	–	–	–	–	–	–
52	–	–	–	–	–	–	–	–	–	–	–	–
11	–	–	–	–	–	–	–	–	–	–	–	–
44	–	–	–	–	–	–	–	–	–	–	–	–
58	+++	+++	++	++	++	+	+	+	+	–	–	–
49	+++	+++	+++	++	++	+	+	+	+	–	–	–

* See van Rood (1962).

serum is then retested against fresh leukocytes from the donor who donated the leukocytes for the absorption and against the leukocytes of the other donors in the experiment. In the cross-absorption experiment shown in Table 1, no evidence was found for the presence of antibodies with more than one specificity; all leukocytes which were agglutinated by the serum removed all detectable agglutinins. However, the leukocytes of donor no. 44, although not agglutinated by the serum, did absorb the antibody. In this way, the existence of the agglutination-negative-absorption-positive phenomenon was found. Approximately 10 percent of the leukocyte samples which give a negative reaction with serum no. 36 in the agglutination test can by absorption be shown to carry the corresponding antigen. Obviously, this is of importance when this serum is used for genotyping.

Let us now address the question: How many samples are needed to obtain in a cross-absorption experiment reasonable certainty that the serum under study does not contain two antibodies, one recognizing the antigen M (ain), the other, the antigen E (xtra)? This problem belongs to the field of estimation theory in statistics. It has been discussed in detail elsewhere (van Leeuwen, Eernisse, and van Rood, 1964).

If this question is translated into the language of the statistician, it will read like this: The donors who provided the leukocytes for the cross-absorption experiment may be considered as a random sample of the population. If n is the number of donors used in the cross-absorption experiment, and x is the number of donors of this lot of whom the leukocytes are agglutinated by the extra antibody anti-E, then the fraction $p = x/n$ will give us an impression of the order of magnitude of P, which is the fraction of the population of which the leukocytes carry the antigen E. If $p = 0$, which means that no antibody anti-E was found in the cross-absorption experiment, and $n = 10$, we can find in the appropriate table that the 95-percent confidence interval of P is 0 to 0.31. It is obvious that the greater n is, the more accurate our estimate of P will be. Table 2 summarizes the 95-percent confidence intervals of P for various values of n, when $p = 0$. The meaning of such a confidence interval is, that if a large number of samples is drawn, 95 percent of these intervals may be expected to be correct, that is, to include the value of the parameter P. The upper limit of this interval indicates the maximum value of P which is still possible when $p = 0$.

Until now, 25 sera have been investigated by cross-absorption studies. These sera did not represent random samples. Either they recognized a known leukocyte group or they were thought to contain antibodies directed against one antigen. Nevertheless, 12 of the 25 sera proved to contain antibodies directed against at least two different isoantigens. As these sera represent a selected sample, it is reasonable to assume that more than 50 percent of the random sera with leukocyte agglutinins will contain antibodies of more than one specificity.

III. *The determination of the genotypes.* When a serum with possibly one single specificity has been found, considerable in-

TABLE 2*

95% Confidence Limits of P for $p = 0$ and Various Sample Sizes (n).

n	P
10	0 — 0.31
12	0 — 0.27
16	0 — 0.21
24	0 — 0.14
40	0 — 0.09
100	0 — 0.04
1000	0 — 0.004

P = the fraction of the population of which the leukocytes carry the antigen recognized by an extra antibody.

n = number of leukocyte samples used in cross-absorption experiments.

$p = 0$ means that in the cross-absorption experiment no evidence was found for the existence of an extra antibody.

* See van Leeuwen, Eernisse, and van Rood (1964).

formation on this serum should be acquired before it is used for genotype determinations. Such information should answer the following questions: Does it give agglutination-negative-absorption-positive reactions, as is the case for most sera? What is the percentage of weak reactions? Is there, regardless of the cross-absorption experiment, still the suggestion of an extra antibody? etc.

Reproducibility is generally not an important problem, if the selected sera give few dubious reactions.

The agglutination-negative-absorption-positive samples and the extra antibodies provide greater difficulties. The agglutination-negative-absorption-positive samples can, as the term denotes, be classified only by absorption. This means that all negative samples obtained with a serum which gives many agglutination-negative-absorption-positive reactions have to be absorbed, which represents a laborious procedure. The extra antibodies are especially cumbersome when they recognize an antigen E, which is rarely present in the absence of the main antigen. Its detection can be quite difficult, if the frequency of the antigen E is low.

Even if one uses strong sera, one will encounter 2 to 4 percent of the donors whose leukocytes will give only weak reactions, even with the strongest sera. Here again, one has to rely on absorption. The mechanism of this phenomenon is not understood. It appears to manifest a familial incidence and therefore might be genetically determined.

More frequent is the occurrence of a weak reaction with the anti-"a" sera and a strong one with the anti-"b" sera or *vice versa*. This occurs in about 10 percent of the leukocyte samples. This can be especially misleading if one uses a small number of identical sera, leukocyte samples of "a+b+" type erroneously being classified as "a+b−" or "a−b+".

Group Four

The data on group Four have been published before (van Rood, van Leeuwen, and Bosch, 1961; van Rood, 1962; van Rood and van Leeuwen, 1963). Only a few additional random samples (Table 3) and families (Table 4) have been tested. The new data are in agreement with the former.

So far 15 women with anti-4*a* and 15 women with anti-4*b* antibodies have been found (Table 5). Seven of the anti-4*b* sera were reasonably good, four gave weak reactions, and four contained, apart from the anti-4*b*, another antibody. In Table 5, the results of the first seven sera with the leukocyte samples nos. 11, 27, 30, 53, and 56 merit special attention. Although the sera were reasonably strong, the majority of the reactions were negative, but by absorption the samples could be shown to contain the antigen in question. It remains a possibility that these agglutination-negative-absorption-

TABLE 3*

Expected and Observed Frequencies of the Phenotypes of Group Four in a Group of 347 Random Donors

Observed	*Numbers*	*Fraction of total*
4 (*a*+*b*−)	46	0.1326
4 (*a*+*b*+)	175	0.5043
4 (*a*−*b*+)	126	0.3631
Total	347	
	Gene freq. 4*a* = 0.3847	
	Gene freq. 4*b* = 0.6153	

Expected	*Numbers*
4 (*a*+*b*−) = $0.3847^2 \times 347$	= 51.35
4 (*a*+*b*+) = $2 \times 0.3847 \times 0.6153 \times 347$	= 164.27
4 (*a*−*b*+) = $0.6153^2 \times 347$	= 131.37
$\chi^2 = 1.478$ $0.20 < p < 0.30$	

* See van Rood, and van Leeuwen (1963).

TABLE 4*

Distribution of Leukocyte Group Four in Family Studies

Mating	Children			
	No.	4(a+b−) no.**	4(a+b+) no.**	4(a−b+) no.**
4(a+b+) × 4(a+b+)	9	10 (11¼)	24 (22½)	11 (11¼)
4(a+b+) × 4(a−b+)	11	0 (0)	16 (18)	20 (18)
4(a+b+) × 4(a+b−)	5	6 (9½)	13 (9½)	0 (0)
4(a+b−) × 4(a−b+)	4	0 (0)	19 (19)	0 (0)
4(a+b−) × 4(a+b−)	0	− (−)	− (−)	− (−)
4(a−b+) × 4(a−b+)	4	0 (0)	0 (0)	21 (21)
Total	33	16	72	52

* See van Rood, and van Leeuwen (1963).
** The expected values are given in parentheses.

positive reactions reflect an antigenic variation of the antigen 4*b*.

The conclusion which can be made from this observation is evident: even the use of three or more specific sera does not guarantee that the results have not been influenced by the agglutination-negative-absorption-positive phenomenon. It also underlines the necessity that, before using a serum for genotyping, information concerning the frequency of agglutination-negative-absorption-positive reactions with that serum should be obtained.

Group Five

In previous publications we have presented data which indicated the existence of a leukocyte group with two alleles, independent of group Four (van Rood, 1962; van Rood and van Leeuwen, 1963). The study of this group was slowed down, because one of the sera, the anti-5*a*, was shown to contain more than one antibody. Closer inspection of the agglutination pattern of this serum with the panel suggested that the anti-5*a* might be contaminated by a weak anti-6*c* (*vide infra*). A cross-absorption experiment with leukocyte samples from donors selected for their 5*a* and 6*c* genotypes proved this (Table 6). As this is the only anti-5*a* serum available, it has to be purified by absorption before it can be used for genotyping.

Table 7 shows the expected and observed frequencies of the phenotypes of group Five on a group of 500 random donors. There is a very good agreement between the expected and the observed frequencies.

Thirty matings with 129 children were also studied. There were no exceptions to the Mendelian laws, and a reasonably good agreement between the observed and expected frequencies was found (Table 8).

Table 9 shows again a part of the panel. Only one anti-5*a* serum is available. On the other hand, four anti-5*b* sera were found: one woman died, in another the antibody disappeared miraculously during the second trimester of pregnancy. Sera nos. 133 and 862 have still retained their original activity. Here, too, agglutination-negative-absorption-positive reactions with serum nos. 14 and 46 were found, though both were good, strong sera. Sera nos. 133 and 862 have been tested against the leukocytes of more than 100 random donors. They are the only two sera in our collection which are so far completely identical. It is noteworthy in this context that these women had their last immunizing stimulus 10 to 35 years ago.

Group Six

The computer had indicated that 13 sera might recognize one group (see page 22). The recognition of the place of these sera in the system of group Six has offered some difficulties. These difficulties will be understandable after inspection of Table 10 which shows the agglutination pattern of these sera with the panel in the numerical order of sera and donors. Every vertical column represents the reaction of one serum with the leukocytes of 100 random donors, and every horizontal row indicates the results of all sera with one leukocyte sample. A black dot means the agglutination was positive, and a blank means the agglutination

TABLE 5
Fifteen Sera with Anti-4*b* Specificity

Genotype	*Donor no.*	*Serum no.*														
		12	*18*	*23*	*48*	*91*	*110*	*790*	*19*	*73*	*115*	*746*	*15*	*59*	*82*	*480*
4 (*a*+*b*−)	8	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	25	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	26	−	−	−	−	−	−	−	−	−	−	−	−	+	++	−
	28	−	−	−	−	−	−	−	−	−	−	−	++	−	(+)	−
4 (*a*+*b*+)	11	−	−	−	−	−	+	+	−	−	−	−	+	−	+	+
	27	−	−	−	−	(+)	+	−	−	−	−	−	−	++	(+)	+
	30	−	−	−	−	−	+++	++	−	−	−	−	−	−	+	+
	56	−	−	−	−	+	+++	++	−	+	−	−	−	−	−	−
	53	−	−	−	+	++	++	++	−	−	−	−	−	+	−	++
4 (*a*+*b*+)	15	+	++	+	++	+++	++++	++++	−	++	+	+++	++	++	++	+++
	23	++	+++	+	++	++	++	++	++	−	−	+	+	+	++	+++
	63	+++	+++	+	++	++	+++	++	+	++	−	+	++	+	+++	++
	69	++	++	++	++	++	+++	+++	+	++	+	−	++	+	+	+++
4 (*a*−*b*+)	1	++	+++	+	++	+++	+++	++	+	++	−	++	+	+	+++	+++
	9	+++	++	+	++	++	+++	+++	+	+++	(+)	+	+	++	+++	++
	13	++++	+++	+++	+++	+++	+++	++	++	++	(+)	++	+	++	+	+++
	45	+++	+++	+	++	+++	++++	++	+	+	(+)	+	+	+	++	+++

TABLE 6*

Cross-Absorption Experiment with Serum No. 4

		Agglutination reaction of the leukocytes from donor no.:											
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
Before absorption		+++	+++	+++	(+)	++	(+)	+++	+++	+++	−	−	−
Genotype		5 (a+) 6 (c+)			5 (a−) 6 (c+)			5 (a+) 6 (c−)			5 (a−) 6 (c−)		
After absorption with leukocytes from donor no.:													
5 (a+) 6 (c+)	1	−	−	−	−	−	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−	−	−	−	−	−
5 (a−) 6 (c+)	4	+++	+++	+++	−	−	−	+++	+++	+++	−	−	−
	5	+++	+++	+++	−	−	−	+++	+++	+++	−	−	−
	6	+++	+++	+++	−	−	−	+++	+++	+++	−	−	−
5 (a+) 6 (c−)	7	(+)	+	+	(+)	+	(+)	−	−	−	−	−	−
	8	+	+	(+)	(+)	+	(+)	−	−	−	−	−	−
	9	+	+	+	+	+	(+)	−	−	−	−	−	−
5 (a−) 6 (c−)	10	+++	+++	+++	(+)	++	(+)	+++	+++	+++	−	−	−
	11	+++	+++	+++	(+)	+	(+)	+++	+++	+++	−	−	−
	12	+++	+++	+++	(+)	+	(+)	+++	+++	+++	−	−	−

* See van Leeuwen, Eernisse, and van Rood (1964).

TABLE 7*

Expected and Observed Frequencies of the Phenotypes of Group Five in a Group of 500 Random Donors

Observed	*Numbers*	*Fraction of total*
5 (a+ b−)	18	0.036
5 (a+ b+)	145	0.290
5 (a− b+)	337	0.674
Total	500	

Gene freq. $5a = 0.181$
Gene freq. $5b = 0.819$

Expected	*Numbers*
$5(a+b-) = 0.181^2 \times 500$	16.4
$5(a+b+) = 2 \times 0.181 \times 0.819 \times 500$	148.2
$5(a-b+) = 0.819^2 \times 500$	335.4
$\chi^2 = 0.239$	$0.70 > p > 0.50$

* See van Leeuwen, Eernisse, and van Rood (1964).

TABLE 8*

Distribution of Leukocyte Group Five in Family Studies

Mating	No.	Children		
		5 (a+b−) no.**	5 (a+b+) no.**	5 (a−b+) no.**
5 (a+b+) × 5 (a+b+)	3	5 (3¾)	4 (7½)	6 (3¾)
5 (a+b+) × 5 (a+b−)	1	0 (1½)	3 (1½)	0 (0)
5 (a+b+) × 5 (a−b+)	11	0 (0)	22 (23½)	25 (23½)
5 (a−b+) × 5 (a+b−)	0	0 (0)	0 (0)	0 (0)
5 (a−b+) × 5 (a−b+)	15	0 (0)	0 (0)	64 (64)
5 (a+b−) × 5 (a+b−)	0	0 (0)	0 (0)	0 (0)
Total	30	5	29	95

* See van Leeuwen, Eernisse, and van Rood (1964).
** The expected values are given in parentheses.

was negative. Table 11 shows the same pattern, but now the leukocytes with the positive reactions are shifted as much as possible to the bottom and the negative ones to the top.

We were not able to fit even now these sera in a di- or tri-allelic system, so we resorted to cross-absorption studies. Cross-absorption experiments with serum 51 showed that it might recognize only one antigen, which we called 6*a*. The cross-absorption experiment with serum 13 gave some difficulties. The first cross-absorption experiment suggested that antibodies with two different specificities were present (Table 12). The agglutination pattern of serum 13, assuming that it contained two antibodies, could, however, not be placed in a logical pattern with the anti-6*a* serum. Furthermore, all the other data suggested that this serum carried antibodies of a single specificity. After some preliminary experiments, the cross-absorption experiment was now repeated: for absorption, leukocytes from 500 ml of blood were used or 10 times the amount employed in the first cross-absorption experiment. Table 13 shows that now indeed the results are consistent with the assumption that only an antibody with one specificity is present, which we called 6*b*. We may conclude from this that the antigen 6*b* has two variants, one weak and the other strong.

In Table 14 the sera are divided into: the "pure" ones, i.e., those recognizing only one antigen, on the left and the "impure" ones on the right. The first, serum 51, recognizes the antigen 6*a*. The next two sera, nos. 13 and 55, recognize the antigen 6*b*. The third group, four sera (nos. 6, 11, 27, and 38), might recognize the strong variant of 6*b* which we called 6*c*. The last group comprises anti-6*b* and anti-6*c* sera, which were not pure. (The combination of a large number of impure sera with the occurrence of an antigen with two variants explains why the elucidation of this system has offered so many difficulties.)

Table 15 shows the total pure anti-6*a*, -6*b*, and -6*c* sera available to date.

Only a small number of random donors have been tested as yet, but the distribution of 6*a* and 6*b* shows a close fit between expected and observed frequencies of the phenotypes (Table 16). The family studies have not yet been completed, but the 16 matings studied so far also show fair agreement (Table 17).

The anti-6*a* sera are noteworthy for two related reasons:

(1) they give many agglutination-negative-absorption-positive reactions, and

(2) the antibody tends to disappear much more quickly from the serum than, for instance, the antibodies of groups Four and Five.

Other Groups

After the initial 60 sera, another 57 were studied, a total of 117 sera, which can now be classified as shown on Table 18. Forty-eight sera defied classification; on the basis of the leukocyte groups of the women who made some of these antibodies and the leukocyte groups of their husbands, they could not have made an anti-4*a*, -4*b*, -5*a*, etc., and for that reason the sera are recognizing groups as yet unidentified. Preliminary studies suggest that they will recognize at least three different antigens.

TABLE 9*

Results of the Agglutination Reaction of the Anti-5*a* and Anti-5*b* Sera with the Leukocytes of a part of the Panel

Leukocytes of donor no.	*Anti-5a serum no.*		*Anti-5b serum no.*			
	4 unabs.	*4 absorbed*	*14*	*46*	*133*	*862*
58	+++**	+++	−	−	−	−
93	+++	+++	−	−	−	−
60	+++	+++	−	−	−	−
86	++	++	++++	++++	++++	+++
96	++	++	++	+++	++++	+++
78	+++	+++	++	++	++++	+++
17	+++	+++	+	+	++++	+++
8	++	+	−***	−	++++	+++
35	++	++	−	−	++++	++
23	++	++	−	(+)	+++	n.d.
94	++	++	−	−	++++	++
63	+++	+++	−	−	++++	+++
51	+++	+++	+	+	++++	n.d.
49	++	++	++	++	++++	+++
79	+++	+++	+	+	++++	+++
50	++	++	+++	−	+++	+++
61	++	−	++++	+++	++++	+++
75	+	−	+++	++++	++++	+++
72	++	−	++	++++	++++	+++
18	+	−	++	+++	++++	+++
42	+	−	+	+++	++++	+++
32	(+)	−	++	++++	++++	+++
56	+	−	++	++++	++++	+++
34	+	−	++	++++	++++	+++
67	+	−	+++	++++	++++	+++
4	−	−	++	+++	++++	+++
13	−	−	−	++	++++	+++
80	−	−	+	++++	++++	+++
47	−	−	++	++++	+++	+++
64	−	−	+++	+++	+++	++++
1	−	−	+++	++++	++++	+++
27	−	−	+++	++++	++++	+++
89	−	−	+++	++++	++++	++++
41	−	−	++	++++	++++	+++
9	−	−	+++	++++	++++	+++
46	−	−	++	+++	++++	+++
66	−	−	++	++++	++++	+++
25	−	−	+++	++++	+++	+++
87	−	−	++++	+++	++++	+++
7	−	−	+++	+++	++++	+++
100	−	−	+++	++++	++++	+++
22	−	−	+	++++	++++	+++
28	−	−	+++	++++	++++	+++
57	−	−	++	++++	++++	+++
6	−	−	++++	++++	++++	+++

* See van Leeuwen, Eernisse, and van Rood (1964).
** − = agglutination reaction negative
(+) = agglutination reaction weakly positive
+, ++, +++, ++++ = agglutination reaction 1, 2, 3, 4 plus positive
*** Agglutination-negative-absorption-positive (see text).
n.d. Not done.

TABLE 10

Group Six

The agglutination pattern of the sera of group Six with the panel.

■ = agglutination reaction positive.
= agglutination reaction negative.

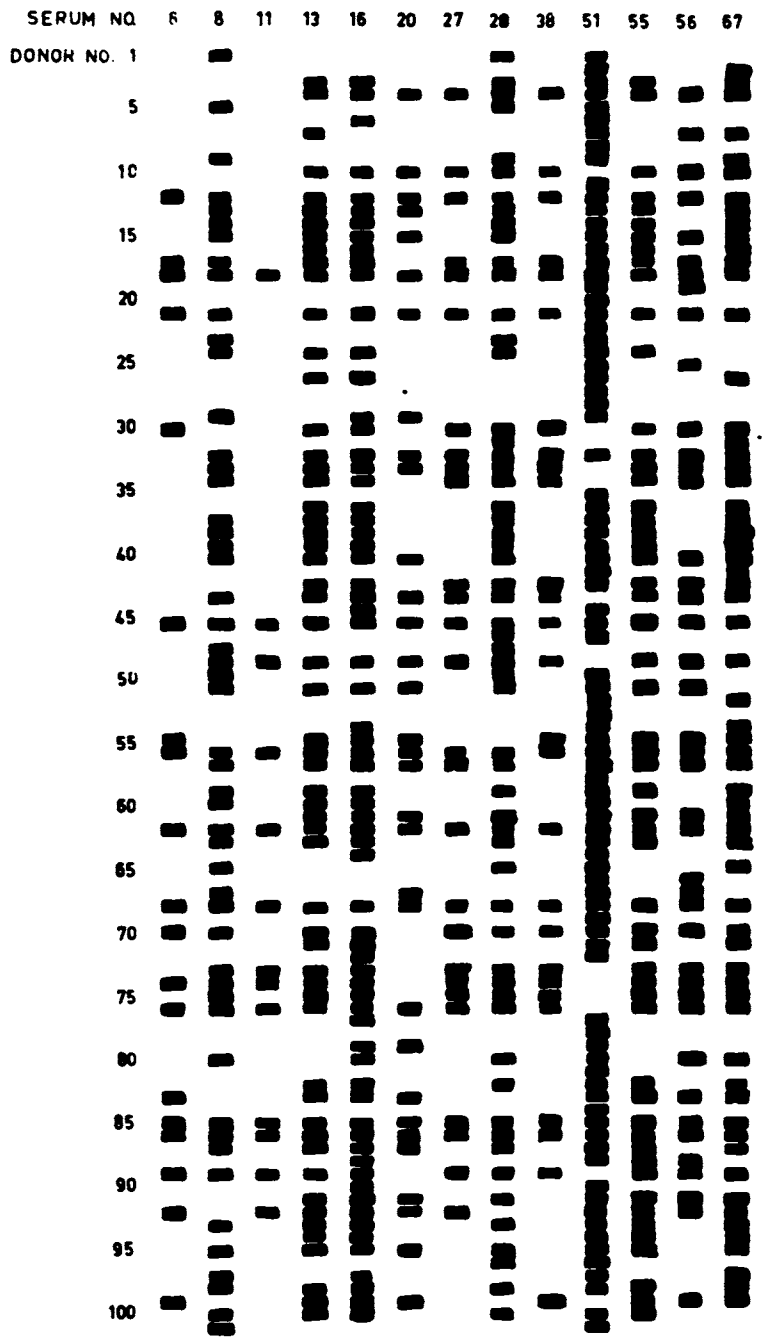
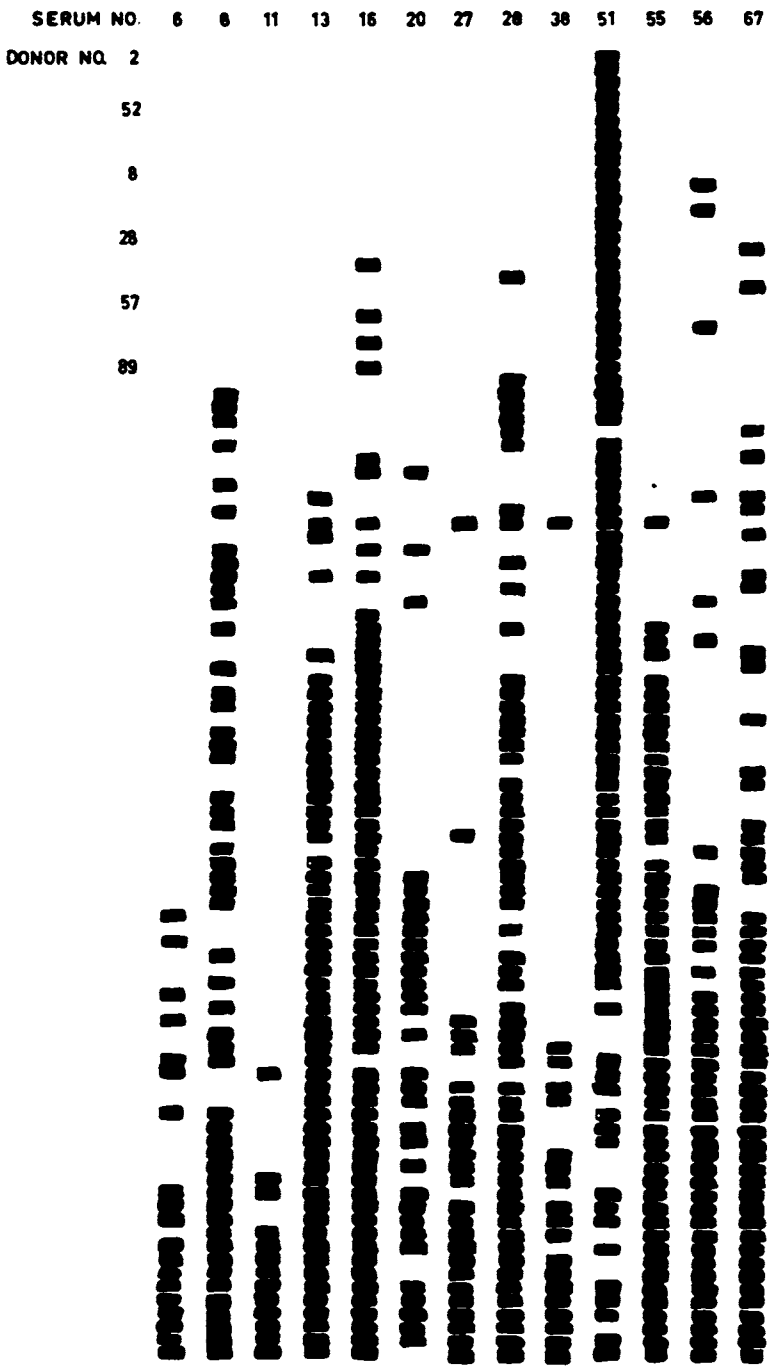


TABLE 11*

Group Six



* Same as Table 10, except that the leukocyte samples with the positive reactions have been shifted to the bottom.

TABLE 12

Cross-Absorption Experiment with Serum No. 13 with 0.25 × 10^9 Leukocytes per ml Serum.

	Agglutination reaction of the leukocytes from donor no.:									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Before absorption	+	+	++	+	+	++	++	++	−	−
After absorption with 0.25 × 10^9 leukocytes from donor no.:										
1	−	−	−	−	−	++	++	++	−	−
2	−	−	−	−	−	++	++	++	−	−
3	−	−	−	−	−	+	+	++	−	−
4	−	−	−	−	−	++	++	++	−	−
5	−	−	−	−	−	++	++	++	−	−
6	−	−	−	−	−	−	−	−	−	−
7	−	−	−	−	−	++	−	++	−	−
8	−	−	−	−	−	−	−	−	−	−
9	+	+	++	+	+	++	++	++	−	−
10	+	+	++	+	+	++	++	++	−	−

TABLE 13

Cross-Absorption Experiment with Serum No. 13 with 2.5 × 10^9 Leukocytes per ml Serum.

	Agglutination reaction of the leukocytes from donor no.:									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Before absorption	++	++	++	+++	++	+	+++	++	−	−
After absorption with 2.5 × 10^9 leukocytes from donor no.:										
1	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−	−
4	−	−	−	−	−	−	−	−	−	−
5	−	−	−	−	−	−	−	−	−	−
6	−	−	−	−	−	−	−	−	−	−
7	−	−	−	−	−	−	−	−	−	−
8	−	−	−	−	−	−	−	−	−	−
9	+	++	++	+++	++	+	++	++	−	−
10	++	++	++	+++	++	+	+++	++	−	−

Discussion

If it turns out that these leukocyte groups can be used in the selection of homograft donors and recipients, an essential question will be how reliable these "typings" are. This will depend, of course, on a number of factors, such as the experience of the investigator, the amount of available time, facilities, etc., but certainly most essential will be the quality of the sera and the knowledge of their limitations. If these two points are kept in mind, and absorption studies are used to complete the results of the agglutination tests, then the determination of the genotypes is reliable and completely reproducible.

If, on the other hand, too few sera are used under suboptimal conditions, quite a

TABLE 14

Group Six

The sera which recognize single antigens have been shifted to the left (nos. 51, 13, 55, 6, 11, 27 and 38). Serum no. 51 is anti-6*a*, sera nos. 13 and 55 are anti-6*b*, and the other sera recognize a variant of 6*b* called 6*c*.

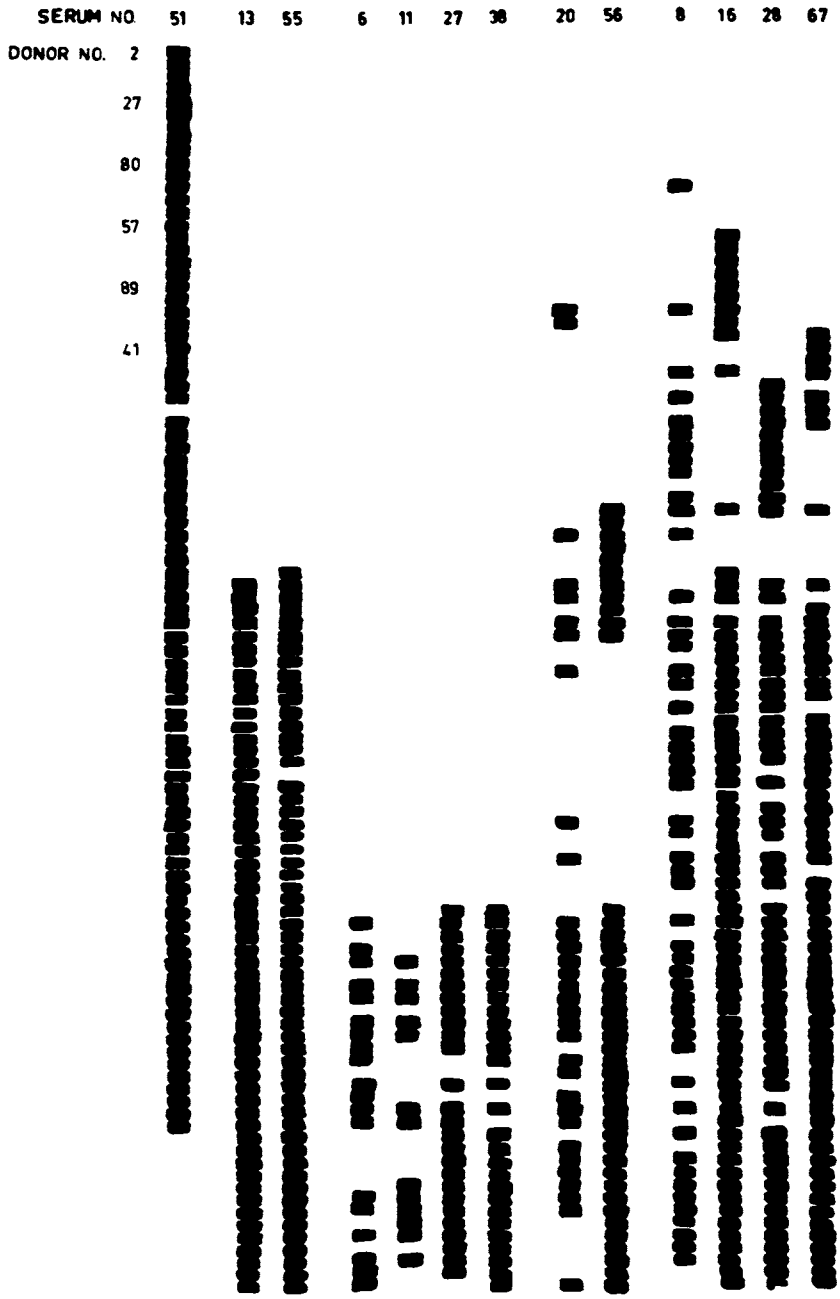


TABLE 15
Group Six

The pure anti-6*a*, anti-6*b*, and anti-6*c* sera so far available.

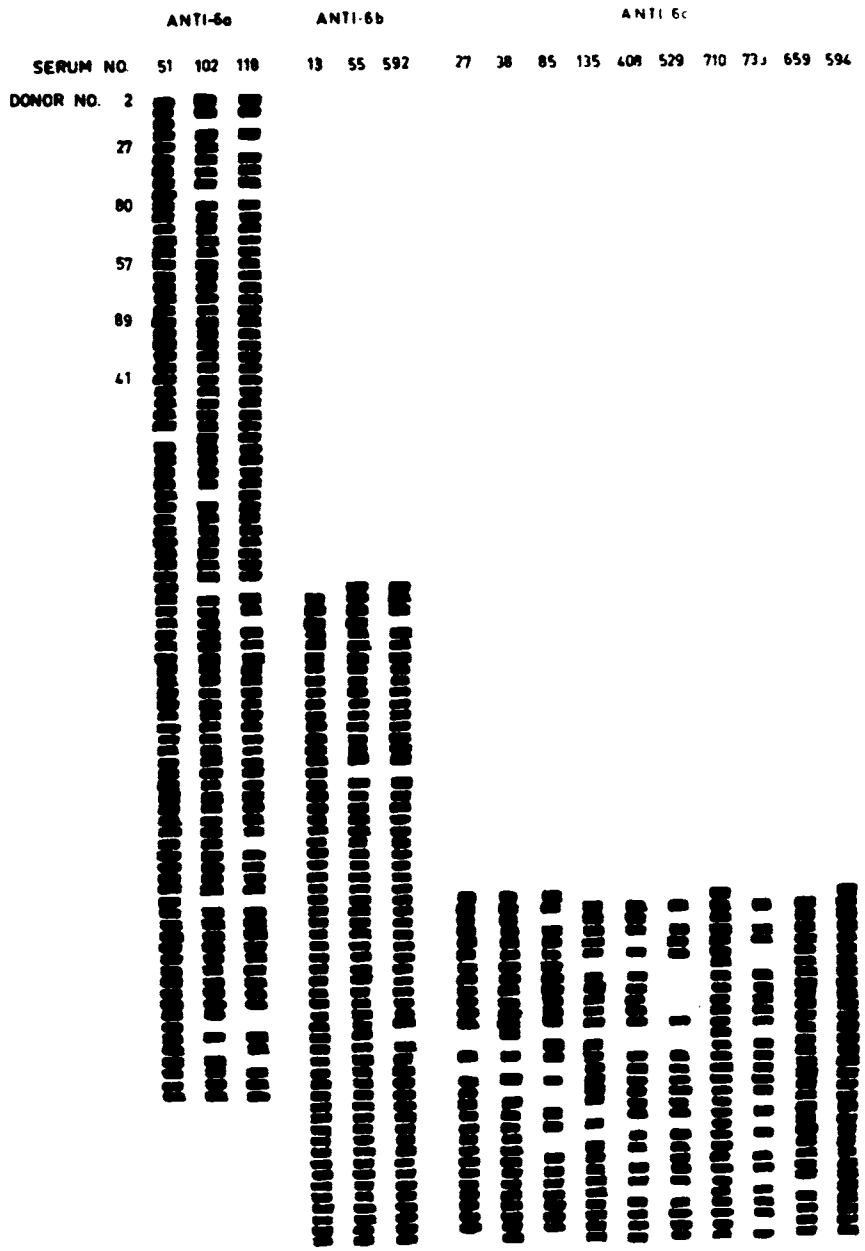


TABLE 16

Expected and Observed Frequencies of the Phenotypes of Group Six (only for the antigens 6*a* and 6*b*) in a Group of 351 Random Donors

Observed	*Numbers*	*Fraction of total*
6 (*a*+*b*−)	151	0.430
6 (*a*+*b*+)	160	0.456
6 (*a*−*b*+)	40	0.114
	351	1.000
	Gene freq. 6*a* =	0.658
	Gene freq. 6*b* =	0.342

Expected	*Numbers*
6 (*a*+*b*−) = $0.658^2 \times 351$ =	151.9
6 (*a*+*b*+) = $2 \times 0.658 \times 0.342 \times 351$ =	157.95
6 (*a*−*b*+) = $0.342^2 \times 351$ =	41.05
	350.90
$\chi^2 = 0.059$ $0.80 < p < 0.90$	

TABLE 17

Distribution of Leukocyte Group Six in Family Studies

Mating	*No.*	*Children* 6 (*a*+*b*−) *no.**	6 (*a*+*b*+) *no.**	6 (*a*−*b*+) *no.**
6 (*a*+*b*−) × 6 (*a*+*b*−)	2	8 (8)	0 (0)	0 (0)
6 (*a*+*b*−) × 6 (*a*+*b*+)	9	17 (19)	21 (19)	0 (0)
6 (*a*−*b*+) × 6 (*a*+*b*−)	1	0 (0)	2 (2)	0 (0)
6 (*a*−*b*+) × 6 (*a*−*b*+)	0	0 (0)	0 (0)	0 (0)
6 (*a*−*b*+) × 6 (*a*+*b*+)	1	1 (1½)	0 (0)	2 (1½)
6 (*a*+*b*+) × 6 (*a*+*b*+)	3	2 (3½)	11 (7)	1 (3½)
Total	16	28	34	3

* The expected values are given in parentheses.

TABLE 18

The Specificity of 117 Different Sera with Leukocyte-Agglutinins

15	anti-4*a*
15	anti-4*b*
1	anti-5*a*
5	anti-5*b*
3	anti-6*a*
11	anti-6*b*
19	anti-6*c*
48	unclassified

significant percentage of the results will prove to be unreproducible. This can be an important handicap when large numbers of donors have to be tested or family studies must be done.

The fact that leukocytes carry the transplantation antigens, of course, does not mean that all isoantigens present on the leukocyte are transplantation antigens. Which antigens will be of importance in homotransplantation and which will not be will have to await further experimentation.

It will in this context be quite interesting to investigate whether the antigens recognized by Shulman *et al.* (1962) with the complement fixation test are the same as the antigens just discussed. A comparison of the results with the agglutination test with those obtained by cytotoxicity tests is in progress. The work of Walford (1964) and Terasaki *et al.* (1964) has shown that also in men the cytotoxicity test recognizes isoantigens.

Summary

Leukocytes are capable of inducing homograft sensitivity. This implies that some of the isoantigens or groups of the leukocyte will be identical with the transplantation antigens. Leukocyte groups can be recognized by means of sera with leukoagglutinins formed during pregnancy. These sera have, however, the disadvantage that they give false negative reactions and often contain antibodies of more than one specificity. A procedure has been reviewed by which these difficulties can be circumvented and by which sera with antibodies recognizing a single antigen can be selected. With the aid of such selected sera, three different leukocyte groups could be recognized: group Four with the antigens 4*a* (gene frequency: 0.38) and 4*b* (gene frequency: 0.62); group Five with the antigens 5*a* (gene frequency: 0.181) and 5*b* (gene frequency: 0.819); and group Six with the antigens 6*a* (gene frequency: 0.658) and 6*b* (gene frequency: 0.342) and the variant of 6*b*, 6*c*. Family studies showed the antigens of groups Four, Five, and Six to be inherited as simple autosomal Mendelian codominant alleles. Of 117 sera with leukoagglutinins, 69 could be shown to have either group Four, group Five, or group Six specificity. The others probably recognize isoantigens which belong to different systems.

Acknowledgments

The sera from pregnant women were obtained from the Departments of Obstetrics of the University Hospital in Leiden (Head, Prof. Dr. A. J. M. Holmer) and the St. Elisabeth Hospital in Leiden (Dr. R. T. P. Niemer). We would like to thank Prof. Dr. Holmer and his staff and Dr. Niemer for their cooperation. Our acknowledgments are also due to Dr. H. de Jonge for his advice on the different statistical problems.

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Discussion

DR. CEPPELLINI: First of all, I want to express to Dr. van Rood and his collaborators my admiration for their excellent work. The statistical approach to recognition of different degrees of relationship between antileukocyte sera has represented a fundamental new opening into the field of leukocyte immunogenetics.

As a geneticist, however, I want to make a remark. You have often spoken about *genotyping* in reference to the results of serological reactions when using the antisera supposedly detecting allelic antigens. In my opinion the correct word should be *phenotyping*; in fact, you are describing serological phenotypes, and equating phenotype with genotype is permissible only in special cases, for instance when the existence of two and only two alleles has been clearly established. The simple genetic situation of system 4 possibly, I would say most likely, will not hold for other systems of leukoantigens. We should probably expect more complicated situations, for instance similar to the Rh factor, where "partial" antigens are the products of complex genes and may be transmitted either in coupling or in repulsion.

Thus I think that the word genotyping should be reserved to the results of formal genetic analysis, that is, family data.

Finally, I must compliment you again for having identified two more systems. It is rewarding to see that good genetic data can also be extracted from leukocyte serology.

DR. van ROOD: In regard to my use of the word "genotyping," I see your point and agree that it is desirable to have a clear distinction between genotype and phenotype.

Regarding the question of the probability that an extra antibody might be present, though it did not demonstrate itself in the cross-absorption experiment: I am unable to explain to you the details of the statistical method involved. However, you will agree with me that the leukocyte samples used in the cross-absorption experiment represent a random sample of the total population. The larger the sample, the more representative of the population it will be, as determined by simple statistical relationships. Although the general principle of our statistical considerations holds, I will agree with you that this reasoning is an oversimplification, because it does not take in account all information based on serological and genetical considerations.

DR. PAYNE: I should like to join in congratulating Dr. van Rood on his pioneer studies on the delineation of three human leukocyte blood groups. After 2 years of exchange of material with him, during which many serologic difficulties were encountered, we are now happy to report tentative confirmation of his first leukocyte antigen system, group Four. Although anti-4*b* sera were readily identified, anti-4*a* sera were not easily detected in the population of our area. For the present we would tend to agree with him that group Four appears to be a two-allele system, but the possibility of additional alleles or components has not been excluded.

I should like to tell you, in brief, about progress in our laboratory on the search for leukocyte blood groups which was presented at a recent meeting on human genetics. The detailed procedures and findings will shortly appear in the report of the Cold Spring Harbor Symposium on Quantitative Biology (Payne *et al.*, 1964). The substance of our report is the description of the discovery of a new leukocyte antigen system, independent of group Four, which consists of three (or more) alleles.

White-blood-cell suspensions were prepared from defibrinated blood, a technique which

gives rise to a more sensitive agglutination test than when leukocyte suspensions are prepared from blood drawn into sequestrene. In working with the group Four antisera, we compared data obtained by our technique with the sequestrene-type preparation utilized by Dr. van Rood. The analytic procedure which we employed to interpret the agglutination reactions of our leukocyte antisera is an extension of that which he used. It permits analysis of the reactions of multispecific sera which are the predominant form obtained from previously pregnant women. This source, it should be emphasized, which often yields multispecific antisera, nevertheless provides less complex sera than those gathered from multitransfused patients.

In our search for leukocyte blood groups, the agglutination reactions of 35 antisera with cells from 150 to 200 individuals were summarized in the form of 2 × 2 tables. An IBM 7090 computer was used to sort the data, to calculate a 2 × 2 chi-square (with Yates's continuity correction) for each association, and to indicate whether the association was positive or negative. Initially 19 of the sera were resolved into three categories which were such that all (or nearly all) of the sera within a category showed significant pair-wise associations. The 2 × 2 chi-squares for these sera are shown in Table 1. These three categories of antisera were interpreted as defining three different agglutinins and hence three corresponding antigens, one agglutinin common to each category of antisera. Individuals carried the antigen if they reacted to all the sera in a category. Almost all the pairs of sera consisting of one from the first category (anti-LA1)* and one from the second category (anti-LA2) showed a negative association which was significant about half the time. The small values involving sera 30 and 32 were in large part due to the small number of tests carried out with these. The negative association implies that the antigens LA1 and LA2, defined by the two corresponding categories of shared antibodies, are determined by allelic (or very closely linked) genes. In all matings involving women who produced the antisera, the combined phenotypes of husband and wife fit with the specificity of the serum produced.

Since there is an appreciable number of people who do not react to sera from either category, anti-LA1 or anti-LA2, there must be at least one more allele in this system. Some effort has been devoted to identifying a serum containing the antibody corresponding to the third allele, but so far without success. Absorption studies indicate that two of our antisera defining this system may be monospecific, one identifying LA1 and the other identifying LA2. The antigens LA1 and LA2 were also shown by absorption of the antisera to be present in granulocytes, lymphocytes, and platelets, but not in erythrocytes.

The population frequencies and familial segregation of the four leukocyte types in the LA system, LA1+ LA2+, LA1+ LA2−, LA1− LA2+, and LA1− LA2−, are interpreted on the basis of three autosomal codominant alleles *LA1*, *LA2*, and *LA3*, with gene frequencies of 0.16, 0.28, and 0.56 respectively (Table 2).

The third group of sera identified van Rood's antigenic determinant 4*b* and three additional sera outside the main categories contained anti-4*a*. Pedigree data and population frequencies indicate that the LA and the 4 systems are unrelated.

Of the 35 antisera with which these studies were initiated, 22 were found to identify antigens of either the LA or the group Four systems. A most encouraging observation was made with respect to the 13 unclassified antisera. Each of the 13 appeared to be related to a component of one of the other sera, indicating that there were a limited number of antigens that might be defined by sera from parous women. Whether the number of loci involved in these leukocyte systems, as questioned by Dr. Brent, will be 50 or 20, we are unable to say as yet. Whatever their number, the elucidation of the three two-allele systems by Dr. van Rood and the single three-allele system by Dr. Bodmer and myself, brings us a long step forward in the understanding of what Race and Sanger chose to designate the "so splendidly complicated antigens of the white cells" (Race and Sanger, 1958). Undoubtedly, as more of these new antigen systems are explored, the analysis of additional systems will proceed at an ever-increasing rate. Before long, it will be possible to evaluate what role, if any, the leukocyte-antigen systems defined by the agglutinins play in transplantation.

DR. van ROOD: In the first place I would like to congratulate you, Dr. Payne, on your system.

As you know, the computer has been extremely helpful to us and I am glad to hear

* L = leukocyte; A = locus; l = allele.

TABLE 1

X^2 Relationships of LA1 and LA2 Sera

Serum Number		2	8	14	15	25	30	32	6	7	9	17	28	22
	2													
	8	*43*												
	14	*69*	*37*											
LA1	15	*42*	*39*	*47*										
	25	*6*	*12*	*10*	*9*									
	30	*24*	*20*	*20*	*25*	*8*								
	32	*18*	*12*	*22*	*8*	*2*	*15*							
	6	*9*•	*11*•	*18*•	*10*•	*9*•	1•	.2•						
	7	*11*•	1•	*12*•	4•	2•	0•	0•	*28*					
	9	*5*•	*5*•	*14*•	*10*•	*9*•	0•	0	*40*	*32*				
LA2	17	1•	1•	3•	6•	.2•	.2	0•	*18*	*19*	*17*			
	28	4•	4•	3•	*13*•	*10*•	.6•	3	*40*	*15*	*20*	*16*		
	22	3•	*12*•	*9*•	4•	.6•	0	.2	*24*	*12*	*9*	*19*	*41*	
Serum Number		2	8	14	15	25	30	32	6	7	9	17	28	22
				LA1					LA2					

X^2 Relationships of 4*b* and putative 4*a* Sera

Serum Number		3	10	11	18	19	26	4	31	33
	3									
	10	*28*								
	11	*19*	*36*							
4*b*	18	*11*	*34*	*35*						
	19	4	*35*	*29*	*56*					
	26	2	4	3	*11*	*26*				
	4	0•	2•	2•	5•	5•	.5•			
Putative 4*a*	31	.1•	2•	1•	4•	*10*•	3•	1		
	33	.2	.1•	3•	6•	6•	5•	.5	*9*	
Serum Number		3	10	11	18	19	26	4	31	33
				4*b*				Putative 4*a*		

bold type $X^2 > /5$

• negative association

that Dr. Payne is using it profitably, also. I would like to add that it is probably possible to program the computer further, so that it can analyze a group of sera such as those of group Six.

It is reassuring that Dr. Payne has similarly found that many of the sera have multiple antibodies. I would like to suggest, however, that the anti-4*b* sera might only seem to be purer than the anti-4*a* sera, because they agglutinate a higher percentage of the population. For that reason an extra antibody will be more easily covered up by the main antibody. Therefore, and here I am again in complete agreement with Dr. Payne, it is quite important to test the sera against a large panel.

DR. LALEZARI: In the past few years, we have had in our possession an antibody which we have determined to be monospecific, and we call it anti-"Ke". Comparison with Dr. van Rood's material indicates that the antibody may be identical with or closely related to anti-5*b*. Our studies also indicate that the antigen is present in lymphocytes, monocytes, neutrophilic and eosinophilic granulocytes, and platelets, but it is absent from red cells. The antibody does not fix complement, induce clot-retraction inhibition, or cause serotonin

TABLE 2

Familial Segregation of LA1 and LA2

Parental Types				*Number of Families*	*Total Offspring Typed*	*Offspring Types*			
LA1	*LA2*	*LA1*	*LA2*			*LA1 LA2* + +	*LA1 LA2* + −	*LA1 LA2* − +	*LA1 LA2* − −
+	+	−	+	2	1	0	0	1	0
+	+	−	−	2	9	0	6	3	0
+	−	−	+	10	11	6	4	0	1
+	−	+	−	1	5	0	4	0	1
+	−	−	−	5	10	0	7	0	3
−	+	−	+	7	9	0	0	9	0
−	−	−	+	7	20	0	0	9	11
−	−	−	−	5	5	0	0	0	5

LA Phenotype and Gene Frequencies

Phenotype			
LA1	*LA2*	*Observed*	*Expected*
+	+	11	12.3
+	−	29	27.8
−	+	54	53.0
−	−	41	41.9
Total.....		135	135.0

Gene Frequencies (and standard errors)

LA1 = .1617 ± .03

LA2 = .2811 ± .023

LA3 = .5572 ± .033

Contingency X^2 testing (negative) association between LA1 and LA2 reactions = 9.7 ($P < 0.5\%$)

X^2 testing goodness of fit of expected phenotype frequencies = 0.227 ($P > 10\%$)

release from platelets. We also have another antigen which may be more interesting. This antigen was involved in the cases of neonatal neutropenia we described a few years ago. From studies of the antibody it appears that: (1) probably a single antigen is detected, (2) the antigen is present only in neutrophilic granulocytes, and absent from eosinophiles, lymphocytes, monocytes, platelets, and red cells, as demonstrated by agglutination, antibody-absorption, and elution methods. These *in vitro* data are in complete agreement with the laboratory data derived from the affected infants who had normal lymphocyte, monocyte, eosinophile, and platelet counts despite virtual absence of neutrophilic granulocytes. I think it is most intriguing that there is an antigenic difference between neutrophilic and eosinophilic granulocytes. These antigenic differences might have physiological and biological significance. Dr. Payne and others have raised the issue as to why neonatal neutropenia is so rare. The finding that the antigen involved is limited to neutrophilic granulocytes might offer an explanation: in this particular antigen-antibody system, the total body antigen available to react with and neutralize the antibody is limited. This antibody, therefore, can be more destructive and cause agranulocytosis. To my knowledge, other studied leukocyte antigens are widely distributed; they are present in lymphocytes, monocytes, platelets, and even nonhematological tissues such as placenta. Under these circumstances, the maternal antibodies transmitted to the fetus may be neutralized by elements other than neutrophiles; particularly, they may be "filtered" and made harmless by placental tissue. For further confirmation of these points, we would like to extend our studies to other antibodies reported to have caused neonatal neutropenia.

May I comment on Dr. van Rood's remarks? (1) We have found a leukocyte donor who is 4*a* negative — 4*b* negative. I would like to hear Dr. van Rood's comment on that. (2) We find that agglutination can always be obtained with prolonged incubation, when cells absorb antibody specifically.

DR. van ROOD: I am not quite certain, Dr. Lalezari, that I did get your question correct: you found by agglutination a 4*a* negative — 4*b* negative sample? Now did you or did you not do an absorption experiment?

DR. LALEZARI: We did not perform absorption tests.

DR. van ROOD: Well, this ought to be done. I mean this was exactly the point I was making when I was showing you the data of Table 5. About 2 to 4 percent of the population will give you this difficulty and unless you have really a rather large number of good strong sera and/or do absorption experiments, you will not be able to classify them.

DR. LALEZARI: The leukocyte donor who was found to be 4*a* negative — 4*b* negative was tested with a panel of antisera made available to us by Dr. van Rood. This donor was the only person who failed to react with all of these antisera. We have not encountered specific absorption without agglutination.

DR. van ROOD: I would like to make one more comment on Dr. Lalezari's presentation. That is, on the distribution of the antigens. The antigens of groups Four, Five and Six have now been shown to be present on leukocytes, platelets, placental tissue, kidney tissue, spleen, and lymph nodes. I won't stick my neck out with granulocytes or lymphocytes, but I think they are on both. We are working on lung and GI tract and they appear also to be present there.

I would like to comment briefly on the question whether the antigens which occur only on platelets or occur only on granulocytes are transplantation antigens and are of importance in the transplantation of, say, kidney. I don't think that an antigen that is only present on granulocytes and is not present on the kidney would be very important in kidney transplantation. I do think, however, that it shows us that it is quite possible that some organs will have organ-specific antigens. The so-called platelet antigens that are only present on the platelets are an example of organ-specific antigens which will be important in the case of platelet transfusion. I think it is very likely that we will find in the future antigens that are kidney-specific.

DR. DAUSSET: Group?

DR. van ROOD: Yes, but only for the kidney. They are present only in the tissues of the kidney. Dr. Hager, in Dr. Merrill's group, has shown a very nice way to isolate these antibodies.

DR. WALFORD: I was particularly interested, Dr. van Rood, in your work with the agglutination-negative-absorption-positive phenomenon. In work that is still quite preliminary we seem occasionally to observe a similar phenomenon with cytotoxic antibodies for lymphocytes. One may at times find a surprising amount of absorption using lymphocytes which do not themselves appear to react with the antiserum being absorbed. Do you think this general phenomenon, to the extent that it may be general, of reaction-negativity-absorption-positivity, denotes quantitative or qualitative differences in these cells as compared with the more usual reaction-positive-absorption-positive cells?

DR. van ROOD: For the moment, I would like to say there are probably two groups of, as we call them, ANAP samples. In the first group you have a true variant of the antigen, for instance the 6b and 6c. The other group is less clear: the ANAP phenomenon will be found with some sera and not with others of the same specificity, the difficulty being that one serum will be ANAP with, for instance, leukocyte samples 1 and 2 but will agglutinate samples 3 and 4, while the next serum will agglutinate samples 1 and 2 but will be ANAP with samples 3 and 4. That this is not the whole story is suggested by the observation that the property of the ANAP phenomenon runs in families. This, of course, does not necessarily imply that this represents a quantitative variation in the antigenic strength: it might well be due to some qualitative, e.g., metabolic, difference of the leukocytes which could be inherited.

So I think that in the first group a purely quantitative difference may be at play, while in the second group it might be either quantitative or qualitative or both.

DR. SHULMAN: Concerning the problem of "reaction-negative-adsorption-positive" cells, this occurs in complement-fixation systems with some antibodies, such as anti-P1GrLy^{c1} (Shulman *et al.*, 1962). The amount of antibody adsorbed by cells that

did not fix complement was less than the amount adsorbed by those that did. The ability to fix complement is related not only to the nature of the antibody, but also to the cellular content and surface distribution of antigen. Most complement-fixing antibodies fix complement with all antigenic cells. Since complement fixation is probably necessary for the cytotoxic action of antibodies, these considerations may be pertinent to the problem of measuring antibodies by cytotoxicity tests.

DR. HIRSCHHORN: Returning to the question of cross-reaction between various types of tissues, I think Dr. Merrill has alluded to an important point. There is one tissue that is common to a number of the tissues that have been mentioned, and that is vascular tissue. There is some evidence from cases of idiopathic thrombocytopenic purpura of a cross-reaction between platelets and vascular endothelium. Cross-reaction between platelets and kidney, for example, could easily not be with kidney but with the vascular tissue of the kidney. This may be related to the vasculitis that Dr. Merrill described. This possibility should be testable by means of fluorescein tagging of antibodies and examining their reaction with vascular endothelium. Such an antivascular antibody could be involved in the rejection of grafts.

DR. van ROOD: I agree with you that this is a possibility. In this connection, it is relevant to mention the work of Dr. Shirley Johnson, who was able to show by electron microscopy that platelets first absorb and subsequently fuse onto the endothelium.

DR. SHULMAN: Can I make one comment regarding fluorescent labeling? It was an insensitive technique when used in an attempt to demonstrate the most avid platelet isoantibodies that we have identified so far (Shulman *et al.*, 1961). We could not see the antibody on platelets with either the direct or indirect technique, although by serologic tests we knew the antibody remained attached to platelets through the necessary washing procedures.

DR. DAUSSET: I should like to present some tabulations which are related to the problem of leukocyte groups and histocompatibility. We have found a system which seems to be very similar to van Rood's *4a/4b* system since it shows almost the same antigen frequency. (Figure 1). It was described with

SYSTEM X^aX^b

Out of 48 sera multiparous women, tested with two leuco-agglutination methods (EDTA, and EDTA + Phenol) it has been found

4 "identical" sera anti-X^a_b
3 "identical" sera anti-X^b

Results of typing of 400 non related individuals

Observed		Expected	
X^aX^a	= 78	X^aX^a	= 78
X^aX^b	= 192	X^aX^b	= 190.5
X^bX^b	= 115	X^bX^b	= 116.5
Inconclusive	= 15		

$\chi^2 < 0.04$

Gene frequency X^a = 0.45
Gene frequency X^b = 0.55

The observed phenotype frequencies show good agreement with those predicted on the basis of the Hardy-Weinberg law.

Figure 1

four identical or almost identical sera for one allele and three for the other allele. I have called them anti-X^a and anti-X^b because up to now its possible correlation with the *4a/4b* system has not been tested. Once we had this system in hand we checked it in order to determine whether it had something to do with histocompatibility.

Figure 2 shows the schema of the experiment carried out with Dr. Rapaport in Paris. We chose two homozygote recipients: one homozygote for X^aX^a and the other homozygote for X^bX^b. We grafted four homozygotes X^aX^a on the right arm of each of them and four homozygotes X^bX^b on their left arm. The results are shown in Figure 3. As you can see, we obtained with the X^bX^b recipient nice differences of almost two days between the compatible and the incompatible donors. You can also notice that this recipient tested with 40 other leukoagglutinating sera of an unknown specificity gave 27 positive reactions.

This means that this recipient must be heterozygote for many systems and therefore cannot be immunized against all corresponding antigens. Thus, the number of incompatibilities, apart from the X^aX^b system, is very low. On the eighth day the incompatible grafts were shrunken and showed infiltration, while there was no infiltration on the compatible ones. One could see the circulation in the capillaries of the compatible grafts and no circulation at all, but

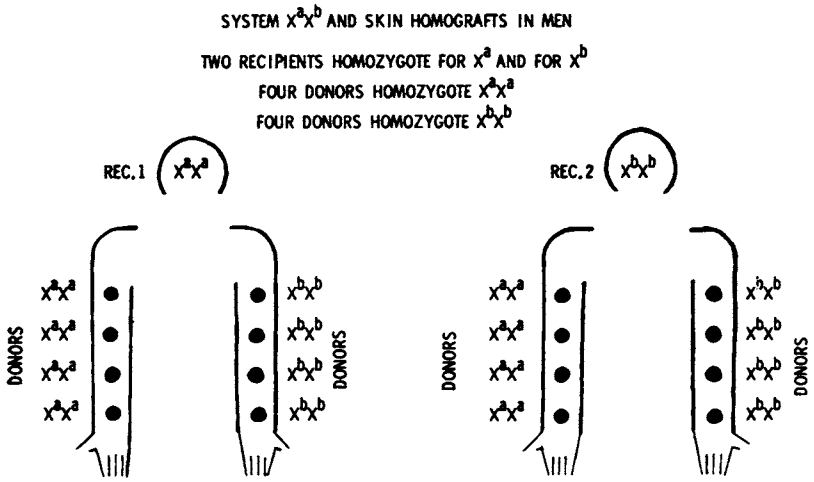

Figure 2

REC. 2 : X^bX^b, Ko+, Zw^b-, PI^{A1}+, BI+ Cl-, EI+

		Other Incompatibilities Leuk	Plat	Rejection (days)
DONORS	X^aX^a	2	1	7
	X^aX^a	0	1	7
	X^aX^a	1	1	7
	X^aX^a	0	0	8

		Incompatibilities Leuk	Plat	Rejection (days)
DONORS	X^bX^b	2	0	9
	X^bX^b	0	0	8,5
	X^bX^b	4	2	9
	X^bX^b	0	1	9

Recipient tested with 40 other leuko-agglutinating sera gave 27 positive reactions

Figure 3

REC. 1 : X^aX^a, Ko-, Zw^{b-}, Pl^{A1+}, $Bl+$, $Cl-$, $El+$

		Incompatibilities Leuk	Plat	Rejection (days)
DONORS	X^aX^a	4	2	10
	X^aX^a	2	1	7
	X^aX^a	5	1	8
	X^aX^a	6	0	9

		Other Incompatibilities Leuk	Plat	Rejection (days)
DONORS	X^bX^b	5	1	9.5
	X^bX^b	2	1	8
	X^bX^b	12	2	9
	X^bX^b	4	1	7

Recipient tested with 40 other leuko-agglutinating sera gave 18 positive reactions

Figure 4

thrombosis, on the incompatible ones. Now we pass on to the X^aX^a homozygote recipient. (Figure 4). It was a failure: there was no correlation between the times of rejection and the X^aX^b system. This may be explained by the fact that this recipient was probably homozygote for many other systems, since he gave only 18 positive reactions when tested with 40 other leukoagglutinating sera. Thus, the number of incompatibilities, apart from the X^aX^b system, is much higher in this case. Therefore, it is very likely that incompatibility in the X^aX^b system was completely masked by these other incompatibilities.

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Serological Activity of Human and Chimpanzee Antisera to Human Leukocyte Isoantigens*

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Risks of sensitizing the recipient and fear of the spread of viral or other diseases limit the usefulness of direct grafting as a means of determining antigenic similarity in man. Alternatives being sought include *in vitro* tests of lymphocyte reactivity, the injection of lymphocytes from a future graft recipient into a series of possible donors to induce a graft-*vs.*-host response, and the use of a third party, the indifferent recipient, as a test subject on which donor and recipient can be compared. None of these methods has the precision of a serological test (and as at present constituted has no standard of reference). It is important to have information on as many individual antigens as possible. There are suggestions from many experimental approaches that a number of weaker antigens can summate to give a response that approximates the reaction of a single strong factor. Certainly in the mouse the strongest incompatibility is exhibited where there is a difference at the H-2 locus together with differences at other nonrelated antigens. H-2 differences alone, or minor antigenic differences alone, give a much weaker response. In terms of suppression of immune response, however, it is simple to overcome the reaction to a series of weak antigens and very difficult to overcome the effects of a very strong one. It is necessary to develop a test that has the potential for recognizing these individual antigens. The role that antigens detected by this test play in graft rejection must be determined, so that not only the degree of incompatibility but also the relative strengths of the particular systems involved can be determined.

Serological typing methods offer not only a precise, safe, and convenient solution, but also a means whereby a large number of potential donors can be matched against a given recipient. Erythrocytes appear to be deficient in many histocompatibility antigens, and fixed tissues, apart from skin, are not readily available from living donors. Leukocytes share many antigens of skin, and will sensitize a graft recipient even more efficiently than a skin graft will (Friedman *et al.*, 1961). The obvious indicator system, therefore, appears to center around the leukocyte.

A serological technique using intact cells as indicator has certain advantages over the use of cell homogenates. Histocompatibility antigens are found in association with the cell surface. Most of the procedures using intact cells as the target do not denature the surface antigens and do not release intracellular enzymes which could destroy or denature the histocompatibility antigens. There is no interference by any intracellular antigens that are not involved in graft rejection. Moreover, some of the surface antigens may not be present inside the cell or may be diluted in the homogenate. The most simple and comprehensive of the techniques using intact cells as antigens is agglutination. A simple and reliable semi-micro method of reasonable sensitivity has been developed which gives reproducibly good controls with normal sera.

The source of antiserum is important. Several workers have found that the sera of recipients of multiple transfusions or of multigravid women contain leukoagglutinins (Dausset, 1954; van Rood, van Leeuwen, and Eernisse, 1959; Payne, 1957). Antibodies can also be produced by experimental immunization. Brittingham (1957) produced high-titered antibodies by the injection of

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leukemic leukocytes into a normal subject and Ceppellini and co-workers (1964) produced antibodies in a proportion of recipients of repeated infusions of whole blood from the same donor. Pilot attempts in our laboratories to produce antibodies by the injection of whole buffy coat subcutaneously and intradermally were unsuccessful. However, potent antisera have been obtained consistently following the intradermal injection of lymphocytes.

Experimental human isoimmunization has risks and limitations. In an attempt to provide an alternative source of potent antisera, recourse was made to heteroimmunization. It was known from the experiments of others that, although immunization of rabbits with human cells led to the production of high-titered leukoagglutinins, the antisera had considerable affinity for species antigens but relatively little isospecificity (Butler, 1960). Immunization within the same order appeared to offer greater promise; for example, Gorer (1938) first demonstrated isoantigenic differences among mice by the use of rabbit heteroimmune sera. Rhesus monkeys were therefore immunized with human leukocytes. Leukoagglutinins were readily produced but, as with the rabbit immune serum, there was no individual specificity. Recourse was then made to chimpanzees which are phylogenetically more closely related to man than monkeys and which are known from the work of Landsteiner and Miller (1925) and others to share at least a number of red-cell isoantigens.

MATERIALS AND METHODS

Antisera

1. Human Isoantibodies

a. *Pregnancy sera.* Sera were collected from the obstetric clinic of Duke Hospital. Samples were taken at intervals during pregnancy, and again shortly after delivery. Samples of clotted blood obtained from the ward or clinic were immediately transported to the laboratory, where the serum was withdrawn and immediately frozen and stored at −20°C until tested.

b. *Experimental isoimmunization.* Antibodies were found in the serum of subjects during the course of other experiments in which injections of purified suspensions of lymphocytes were being given intradermally (Payne and Rolfs, 1958). From a young healthy donor with a history of uneventful blood donation, 100 ml of blood were drawn into a siliconized bottle containing 1000 units of heparin. The blood was passed through 4 g of nylon according to the method of Greenwalt and co-workers (1962). The polymorphonuclear leukocytes (PMN) and platelets remained trapped in the nylon. The red cells were separated from the leukocytes by sedimentation with gelatin as above. The cells obtained after double sedimentation, consisting of 97 to 99.5 percent lymphocytes, were washed and resuspended in saline solution to a concentration of 5×10^7/ml. Then 0.1-ml and 0.05-ml volumes were injected intradermally on the volar aspect of the forearm. Cells from four to eight donors were processed on each day and two injections of each suspension were given to each of the recipients (Amos, Peacocke, and Sieker, in preparation). Subjects were bled at varying intervals after each series of injections. The recipients had been given at least two sets of injections before antibodies were detected; each set of injections consisted of 5.0 or 7.5×10^6 lymphocytes from each of four to eight donors. Seven out of nine immunized subjects have yielded antibodies; one of these subjects had received only two sets of injections totaling approximately 5×10^7 cells. Currently immunizing injections are being given from a single donor to each recipient.

2. Chimpanzee Immunization

a. *Preparation of antigen.* Blood from individual donors of blood groups O or A was collected in EDTA and gelatin,* in the proportion of 10 volumes of blood to 1 volume of 5-percent Na_2EDTA and 2.5 volumes of gelatin. The erythrocytes were allowed to settle, and the supernatant plasma was withdrawn and centrifuged at 4,000 g. The leukocyte-platelet-erythrocyte pellet was then resuspended in Medium 199 containing buffered EDTA. The average inoculum from 200 ml of blood contained 10^9 leukocytes, 3.5×10^9 platelets, and 3×10^9 red blood cells. The cells were transported on ice to the Primate Center at Orange Park and were injected within 12 to 24 hr of collection. Leukocyte viability at the time of injection as determined by dye uptake was 90 percent or greater.

b. *Immunization procedure.* In the initial experiment four chimpanzees were injected intravenously at monthly intervals with the

* Plasmagel—Laboratoire Roger Bellon, Seine, France.

cells from individual human donors. The animals were anesthetized at the time of injection and no transfusion reactions were observed, even when circulating antibody was present.

In a second series of seven chimpanzee immunizations, the animals were first given the leukocyte-rich preparation from 200 ml of donor blood intravenously. Subsequent booster injections were given by four distinct routes: subcutaneously, intradermally in the extremities, intraperitoneally, and intravenously.

Leukocyte Agglutination

The method is described in detail in a separate section of this report. Briefly, blood was collected into 5-percent EDTA, mixed with Plasmagel, and allowed to sediment. The supernatant plasma, heavily contaminated with red cells, was gently centrifuged and the cell pellet resuspended in about 1: 100th the original volume. The red cells remaining in the suspension formed rouleaux at this high cell concentration and sedimented rapidly. The white-cell suspension was then freed from platelets by passing it through a gradient, and resuspended to a concentration of 6×10^6 leukocytes/ml in platelet-free plasma diluted to 3:1 with an EDTA buffer. Leukocyte suspensions tested with chimpanzee serum were freed from platelets by differential centrifugation, rather than by the use of a gradient.

All tests were performed in 6 × 50-mm new or acid-washed siliconized tubes using 0.025-ml volumes throughout.

Antiserum was diluted in a solution consisting of equal parts of EDTA buffer and 0.85-percent saline. Equal amounts of antiserum and cells were mixed and incubated at room temperature for 2 hr. The suspension was gently mixed once, dropped onto a slide, and read microscopically without the addition of acetic acid.

Absorption Procedures

1. Human Sera

A crude preparation of white cells containing red cells and platelets was used for absorption. Blood was collected as above. The supernatant plasma containing most of the white cells was removed and centrifuged at 4,000 g for 20 min. The clear plasma was discarded and the packed-cell button resuspended in a volume of EDTA buffer representing one-tenth the original volume of whole blood. The white cells in this suspension were counted. For complete absorption of 0.5 ml of the most potent sera, four separate absorptions, each with 5×10^7 cells, were required. This procedure has temporarily been adopted as standard. For each absorption a suspension containing a total of 5×10^7 leukocytes was placed in a tube and centrifuged at 4,000 g for 10 min. The supernatant was discarded and 0.5 ml of untreated antiserum was mixed with the packed white cells. The cells were mixed at 5-min intervals for 30 min by aspirating in and out of a pipette. After incubation, the mixture was centrifuged at 4,000 g for 10 min and the supernatant added to a fresh aliquot of packed cells.

After the fourth absorption, the sera were clarified by high-speed centrifugation at 30,000 g for 30 min.

2. Chimpanzee Sera

a. *Erythrocytes.* Chimpanzee serum was inactivated at 56°C for 30 min. Undiluted serum was mixed with an equal volume of packed, washed, pooled human red blood cells. The mixture was incubated at room temperature for 30 min and then centrifuged at 3,000 g. This procedure was repeated until the serum no longer agglutinated human erythrocytes after 1 hr at room temperature when read microscopically.

b. *Leukocyte-platelet mixtures.* Blood was collected in EDTA and gelatin. The platelet-leukocyte-rich plasma was centrifuged at 4,000 g for 10 min. One milliliter of undiluted chimpanzee serum previously absorbed with erythrocytes was added to the cell button from 20 ml of blood. The button usually contained approximately 10^8 leukocytes and 3.5×10^8 platelets.

The mixture was incubated at room temperature for 30 min with intermittent agitation of the tubes and then centrifuged at 30,000 g for 20 min.

Cytotoxicity Test

A modification of the method of Gorer and O'Gorman (1956) using rabbit serum as a source of complement, as recommended by Walford and co-workers (1964), was used. PMN in crude leukocyte suspensions tend to aggregate and to be damaged apparently

nonspecifically by rabbit serum, so purified lymphocyte suspensions have been used in most tests. The rabbit serum was obtained fresh and absorbed twice in the cold, with equal volumes of washed human erythrocytes, then stored in 0.5-ml amounts at −20°C.

The lymphocytes prepared from a nylon column were washed and suspended at a concentration of 2 × 10^6/ml, in barbital buffer containing 0.51 g $MgCl_2$ and 0.11 g $CaCl_2$ per liter. Additional heparin was added to prevent clotting. Equal 0.025-ml amounts of antibody dilution and cells were placed in a 6 × 50-mm tube and incubated for 20 min at room temperature; 0.025 ml of absorbed rabbit serum diluted 1:2 or 1:4 was added to each tube and the mixture incubated at 37°C for 2 hr. Then 0.3 ml of 1:2000 trypan blue in saline was added, the tube centrifuged, and the supernate replaced with a further 0.05 ml of 1:1000 trypan blue solution. Rhesus antihuman leukocyte serum was used as a positive control, normal human serum as a negative control. Results were scored on the basis of trypan blue uptake.

RESULTS

Leukoagglutinating Properties of the Various Types of Antisera

1. Sera from Parous Women

a. *Incidences of leukoagglutinins.* A total of 441 sera have been tested against leukocytes from panels of group O donors (Table 1). As a control, 180 sera from nulliparous, nontransfused donors have been tested. Most of the test sera came from women who had two or more pregnancies. However, 79 were from primipara with no history of transfusion or of abortion, and 54 were from a selected group of women of high parity with a history of bleeding during pregnancy or of other obstetric difficulties. Each serum was tested with at least eight cells from a random panel, all sera were tested at least twice, and in many instances samples have been obtained at intervals during and after delivery.

The frequency with which leukoagglutinins have been detected is shown in Table 1. Only two normal sera produced agglutination; in both instances the reactions were weak and could be detected only at high serum concentrations. This result confirmed those of other investigators (Payne and Rolfs, 1958; van Rood, van Leeuwen, and Eernisse, 1961) and indicates that natural leukoagglutinins are rare in the absence of overt immunization or disease; it also provided an indication that the test was specific and was not unduly sensitive.

The reactions of the sera from primipara are of interest. It has generally been thought that repeated immunization is necessary for the development of antibodies, and in a previous paper (Amos and Peacocke, in press) we showed that there was a correlation between the strength of the leukoagglutinin and the degree of parity of the donor. The sera in Series B (Table 1) are generally of low titer but the reactions are definite. Since antibodies were present before delivery, immunization cannot be simply the result of cells passing into the uterine vessels during the trauma of parturition.

The reactions obtained with sera from multigravida are not exceptional. The incidence is comparable to that obtained by Payne and Rolfs (1958). Series C is composed en-

TABLE 1

Frequency of Leukoagglutinins in Sera from Various Subjects

	*Series A** *Normals (Nullipara, Nontransfused)*	*Series B* *Primipara*	*Series C* *Multipara*	*Series D** *Multipara, Multitransfused*	*Series E** *High Multipara, Aborters, Bleeders*
Pos.	2	7	33	33	35
Neg.	178	72	114	128	19
Total	180	79	147	161	54
% Pos.	1	9	23	21	65

* from data presented by Amos and Peacocke (in press)

tirely of multigravida. Series D, an earlier series, includes a proportion of recipients of blood transfusion. The over-all incidence in Series C and D is 21.4 percent. Series E, also from an earlier series, is in striking contrast to the predominantly healthy populations of Series C and D. The high proportion of positive results in this group suggests that there is a relationship between abnormal pregnancy and the appearance of leukoagglutinins.

b. *Sera from multipara as typing reagents.* For a serum to be useful as a reagent for typing leukocyte antigens it should be monospecific, should give reproducible results, and should, where possible, have a reasonably high titer. The sera found in the multigravida fall into three arbitrary types. Some are extremely weak. These sera often react with cells from relatively few donors and many could be monospecific. The reactions tend to be inconsistent and with present techniques these sera are of little value. An example of such a serum is shown in Table 2; serum M.G., which has been tested on a large panel of cells, reacts with relatively few, and rarely reacts when diluted to more than 1:2.

Sera A.R. and S.H. are included as representatives of sera of moderate reactivity that do give consistent results and could be of value as reagents. Many of these sera react with less than half the donors on a random panel, and superficially might appear to be monospecific. Even such relatively weak sera may contain mixtures of antibody. The reactions of serum A.R., after absorption with leukocytes from two donors, with cells from four selected donors are shown in Table 3. Absorption with cells from donor #0043 removed virtually all activity, but cells #0024 removed only the agglutinin for #0024 and reduced the titer against #0043 only slightly. Cells from donors #0029 and

TABLE 2

Leukoagglutinin Titers of Human Sera with Selected White Cells

Sera	*Test Cells*					
	#0002	*#0013*	*#0029*	*#0031*	*#0036*	*#0034*
M.G.	2	2	0	0	0	0
A.R.	4	0	16	0	0	8
S.H.	8	0	0	2	0	2
G.S.	32	32	32	8	8	32
S.A.	64	16	16	32	32	64

TABLE 3

Absorption of Moderately Reactive Serum A. R. with Selected Cells

*Absorbed with:**	*Initial Dilution*	*Reaction with cells from:*			
		#0024	*#0043*	*#0029*	*#0034*
Nil	Undiluted	+	++	+	++
	½	±	++	+	±
	¼	−	±	−	−
Cells #0024	Undiluted	−	++	++	++
	½	−	±	+	+
	¼	−	−	−	±
Cells #0043	Undiluted	−	−	±	−
	½	−	−	−	−
	¼	−	−	−	−

* All absorption in proportion of 0.5 ml undiluted serum: 5×10^7 leukocytes, four successive absorptions, 30 min at room temperature.

#0034 appeared to be more strongly agglutinated after four absorptions with cells from #0024, a not uncommon finding. This serum obviously contains at least two distinct antibodies.

Sera having a titer of 16 or higher would be extremely useful for typing. Most of these sera are extremely complex and cannot be used without extensive absorption. Some results obtained with serum S.A. are given in Table 4; donor #0002 is the husband. Four sequential absorptions with his leukocytes removed all activity for all cells tested. Absorption with any of the other cells revealed a different final pattern of reactivity. Four such patterns were found but none of the resulting sera appeared to be monospecific. Each could be split into two or more active components displaying additional patterns after absorption with the remaining positively reacting cells.

Nevertheless, it was desirable to determine whether such crudely absorbed sera were capable of at least partially grouping the population. Two of the absorbed sera, therefore, along with the unabsorbed serum were tested against the white cells of 28 random donors. The reactions obtained are shown in Table 5. The cells of four individuals failed to react with the unabsorbed serum of any of the fractions while the remaining 24 gave strong reactions. Four of the remaining cells were not agglutinated by the serum after absorption with cells from subject #0018 or #0020. Nine cells reacted with serum absorbed with cell #0020 but not with cell #0018 and six gave the opposite pattern, reacting with the serum absorbed with #0018 but not the one prepared with cell #0020. The five other cells reacted with serum absorbed with either cell. Even with these crudely absorbed reagents, then, it was possible to divide this population into five separate groups, whereas only two groups (reactors and nonreactors) would be discernible with the unabsorbed reagent.

Further absorptions of this serum indicated that it contains antibodies of at least four different specificities. Monospecific antibodies cannot be prepared by absorption with any single cell known at present, but require separate treatments with two or more different cells. Similar studies with the sera of various multiparous women indicate

TABLE 4

Leukoagglutinin Titers of Serum S.A. after Absorption with Selected Cells

Sera	*Test Cells*				
	#0002	*#0018*	*#0020*	*#0010*	*#0040*
S.A. Unabs.	64	32	32	8	64
S.A. Abs. #0002	0	0	0	0	0
S.A. Abs. #0018	32	0	16	0	32
S.A. Abs. #0020	32	16	0	0	32
S.A. Abs. #0010	32	16	32	0	32
S.A. Abs. #0040	16	0	32	0	0

TABLE 5

Reactions of S.A. Serum Absorbed with Selected Cells, Tested Against Cells From 28 Group O Donors

Unabsorbed S.A.	*Absorbed S.A.*		*Number of Donors in each Class*
	#0020	*#0018*	
0	0	0	4
+	0	0	4
+	+	0	9
+	0	+	6
+	+	+	5

that many which contain potent agglutinins are composed of antibodies having more than one specificity.

2. Sera from Experimental Isoimmunization

Preliminary attempts to induce antibody formation by the injection of large numbers of leukocytes were unsuccessful. In these attempts, white-cell suspensions for immunization were prepared similarly to those used for the immunization of chimpanzees. Cells from a donor of blood group A were injected into one group O and two group A recipients. Injections were given in multiple sites; as many as 200 intradermal inoculations and intramuscular injections were given to a single subject over a 6-month period. Although immune anti-A agglutinins were formed by the O recipient, there were no detectable antileukocyte antibodies reacting with leukocytes from group O subjects in any of the three sera.

Successful immunization came as an incidental finding in another experiment. The lymphocyte transfer test has been suggested by Brent and Medawar (1963) as a possible means of assessing histoincompatibility.

For the test, lymphocytes from a possible graft recipient are injected intradermally into a number of possible donors. The subject showing the least reactivity is regarded as being the most compatible. Our experiments were designed to explore some of the parameters of the reaction, including the change in reactivity that would occur if the test cells came from an immune subject, i.e., from a transfused patient (Amos, Peacocke, and Sieker, in preparation).

Panels of normal subjects were assembled and cells from each member of the panel were injected into other members. The experiment was repeated, with variations, several times over a 3-month period. Many of the original subjects were used in several experiments. Serum samples were drawn and were found to contain leukoagglutinins. The course of immunization differed in two respects from the course used in the deliberate attempt at antibody production. The sensitizing cells from a suspension passed through a nylon column contained between 97 and 99 percent lymphocytes or mononuclear cells and very few platelets; each subject was immunized with the cells from at least five donors.

The total number of cells injected was considerably lower than that in the previous trial, and one subject produced antibodies after receiving two sets of injections totaling only about 5×10^7 cells. To date, sera from nine subjects have been tested. Seven of these contain leukoagglutinins. The frequency of reactions against cells from members of the immunizing panel and against random group O donors is shown in Table 6.

These sera were also found to be cytotoxic. The titers were relatively low with the technique used, most reacting only at a serum dilution of 1:2 or 1:4. Agglutination and cell damage did not show a close parallel when the sera were tested against the same cells by the two methods. Since this may have been a result of a difference in sensitivity of the two techniques, a limited number of absorptions were performed. It was possible to absorb out both the cytotoxic and agglutinating activity for the absorbing

TABLE 6

Reactions of Sera from Isoimmunized Volunteers against Cells from the Immunizing Panel or Cells from Random Group O Donors

Serum from	*Reactions of Cells (positive/negative)*		
	From donor panel	*From random panel*	*Total*
B.H.	3/7	8/12	11/19
D.P.	5/6	8/10	13/16
H.M.	4/6	0/4	4/10
K.P.	4/6	3/7	7/13
A.J.	5/6	2/4	7/10
E.H.	5/7	3/6	8/13
C.M.	4/7	6/12	10/19
Total incidence	30/45	30/55	60/100

cell. In some instances, when this same absorbed antiserum was tested with another cell which gave positive leukoagglutination and cytotoxicity with the unabsorbed antiserum, only the cytotoxic activity remained. Apparently the cytotoxic test will allow the recognition of certain antigens not detectable by agglutination.

3. Sera from Chimpanzees

The results of the chimpanzee immunizations with respect to general types of leukoagglutinins produced and the time the first antibodies were detected are shown in Table 7. In all experiments utilizing leukoagglutination, the chimpanzee sera were previously absorbed with pooled human erythrocytes. Leukoagglutinin titers of the sera absorbed only with erythrocytes ranged from 1:4 to 1:32. Six of the animals produced detectable antibodies and reacted with all human leukocytes tested. Four of these six sera could be absorbed with selected cells and then continued to react with a proportion of other cells in the panel. The remaining two, Homer and Jethrow, produced only one type of antibody that could be removed by absorption with any human cell which showed no isospecificity.

Since most of the sera in the initial tests contained leukoagglutinins which reacted with all individuals tested, leukocyte absorption experiments were performed in order to detect possible isoantigens and to ascertain the number of antigen-antibody systems involved. The reactions of two of these sera, Falweb and Bogam, will be considered in detail.

Absorptions with leukocyte-platelet mixtures were performed as described above ("Materials and Methods"). Table 8 shows the reaction of Falweb antiserum absorbed with leukocyte-platelet mixtures from six different individuals and tested by leukoagglutination with each of the six cells. Each of the six cells gives a different pattern of reactivity with the cell panel. When other cells were used for absorption or for agglutination it was found that they fell into one of these six classes. The minimum number of antigen-antibody systems that could account for these reactions presented in Table 5 is three. The predicted number of antigens on the same individual cells and the antibodies present in the absorbed sera are shown in Table 9. Cell 6 is representative of the leukocyte antigen donor used to immunize Falweb and has all three antigens. Absorption with this cell removes reactivity for all other cells. Thus far in direct testing and absorption experiments we have not encountered a cell containing only antigen 2. All other combinations have been found, including a cell that contains none of the three antigens.

TABLE 7

Development of Leukoagglutinins in Immunized Chimpanzees

Chimpanzee	*Monthly injections*•	*Sera containing leukoagglutinins*••	*Sera reacting with isoantigens*
Initial Series			
Hari	8	0	0
Jethrow	5	+	0
Homer	5	+	0
Falweb	2	+	+
Second Series			
Pandit	1	+	+
Peck	2	+	+
Bogam	2	+	+
Ric	3•••	0	0
Polly	4•••	0	0
Halpha	4•••	0	0
Sue	4•••	0	0

•Number of injections before antibody appeared *or* total injections given if no antibodies present.
••Leukoagglutinin present reacts with all human leukocytes.
•••Immunizations are currently being continued.

TABLE 8

The Reactions of Falweb Serum after Absorption with Various Human Cells

Tested on cells from:	*Serum absorbed with cells from subject:*					
	#0028	*#0014*	*#0030*	*#0012*	*#0045*	*#0038*
#0028	○	+	+	+	+	○
#0014	○	○	○	+	+	○
#0030	+	+	○	+	+	○
#0012	○	+	+	○	○	○
#0045	+	+	+	+	○	○
#0038	+	+	+	+	+	○

+ = Leukocyte agglutination
○ = Negative leukocyte agglutination

TABLE 9

Distribution of Antigens Detected by Absorbed Fractions of Falweb Serum on Various Human Cells

Individual Human Leukocytes	*Antigen on WBC*	*Antibodies in Absorbed Falweb Antiserum*
#0028	1, 3	2
#0014	1	2, 3
#0030	1, 2	3
#0012	3	1, 2
#0045	2, 3	1
#0038	1, 2, 3	—

The sera that had been absorbed with cells from one individual (cells 1, 3, and 5) and were suspected of being mono-specific were then additionally absorbed with each of the remaining cell types with which they reacted. Absorption with any one of the reactors would then remove the reactivity for all. This is a serological proof of mono-specificity. The leukoagglutinin titers of the absorbed sera range from 1:2 to 1:16. The monthly booster injections have not altered the serological specificity of Falweb antiserum. A sample taken 8 months after the first antibodies were detected and then tested by absorption techniques showed the same type of reactivity as the early sample. Moreover, the titers did not increase after the third injection despite efforts to hyperimmunize by altering the dosage and routes of administration.

Another chimpanzee antiserum, Bogam, gave a simpler pattern of reactivity, as shown in Table 10. The antiserum absorbed with red cells but before absorption with leukocytes, reacted with all cells tested. Absorption with leukocytes from different individuals gave only two patterns of reaction.

Cells have been temporarily classified as Bogam Type 1 or Bogam Type 2. Type 1 cells will remove all activity for other Type 1 cells but have agglutinins for Type 2. Type 2 cells will remove all activity. Thus the Bogam serum contained two antibodies, one directed against an antigen common to all human leukocytes, and another directed towards a single isoantigen. Not only was it found that cells from Type 1 would remove activity for all Type 1 cells, but sequential absorption with different Type 1 cells did not affect the reaction with any Type 2 cell. The serum absorbed with Type 1 can thus be regarded as monospecific for Type 2.

Scattered throughout the literature are references to apparent close similarities between unrelated individuals tested against a battery of leukoagglutinating sera. Such similarities may be spurious, as shown by the following example (Table 11). Cells from three indi-

TABLE 10
Reactions of Immune Serum from Chimpanzee Bogam

Individual Human Leukocyte Suspension	*Bogam Antisera Absorbed with Individual Human Leukocytes*		
	Type 1	*Type 2*	*3 (unabsorbed)*
Type 1	0	0	+
Type 2	+	0	+

TABLE 11
Discrimination between Cells from Unrelated Humans with Various Fractions of Absorbed Chimpanzee Serum

Chimpanzee antisera absorbed with individual leukocytes	*Test Cells*		
	#0012	*#0019*	*#0045*
A - Falweb -#0045	0	0	0
B - Falweb -#0030	+	+	+
C - Bogam -#0015	0	0	0
D - Falweb -#0028	0	0	+
E - Peck -#0015	0	+	+

viduals were tested against five chimpanzee antisera absorbed with individual human leukocytes. All three cells gave identical reaction patterns when tested with sera A, B, and C. A fourth reagent, D, would show that cell #0045 was different, while a fifth reagent, E, clearly demonstrated that none of the cells were identical. Extensive typing of this sort, involving the use of large numbers of sera, both monospecific and group-specific, will be necessary before the individual antigens can be assessed in terms of graft rejection.

Discussion

After an era of optimism only slightly affected by the realization that most of the recipients of a renal homograft die as a direct or indirect consequence of a homograft reaction, it has become apparent to even the most radical proponent of immunosuppressive therapy that this form of treatment alone is inadequate. Immunosuppression can be used to damp down a weak reaction but cannot be relied on indiscriminately to suppress an unknown degree of incompatibility without adverse effects on the patient.

There are many approaches to the problems of tissue typing. Leukoagglutination is only one approach, but one which has the great advantage of genetic precision. Within a finite time it should be possible by direct testing in conjunction with absorption tests to type tissues with a precision at least equal to that now possible with red cells. The most urgent need is for large volumes of monospecific typing reagents. Purification of the antibodies is a prerequisite to isolation of the antigens and to assessment of the relative strength and importance of the various tissue isoantigens.

We have explored three different sources of antibody. An additional source, recipients of multiple transfusions, was avoided since such patients are frequently sick or dying. Each of the other three sources has its own peculiar advantages. It appears probable from observations in progress that the chimpanzee is capable of recognizing some of the same antigens which elicit isoantibodies in multigravida. The sources may well be supplementary or either may pick up antigens not detected by the other. Regardless of source, the sera must be checked for specificity by rigid absorption procedures.

For later studies it may not be necessary to have a complete collection of monospecific antibodies, as long as the composition of a given serum is known. The use of antisera of mixed specificity has given satisfactory

results in Rh grouping in man. The mouse with its complex H-2 system may also serve as a model. Most typing of H-2 configurations is done with antisera prepared in a heterozygote to limit the number of antibodies produced, or with antisera partially simplified by absorption. Monospecific sera in the mouse system are rarely available. Certain desirable configurations of antigens do not occur, or are found very seldom. This has rendered it necessary to determine the presence of such antigens as 2.F and 2.M by inference. Several reagents, each containing anti-F or anti-M together with different combinations of other antibodies, are used. This type of analysis is most frequently met with in compound antigenic systems like H-2 or Rh, where genes controlling several antigens are closely linked, or where a complex antigenic product is formed by a multiple allelic gene.

For multispecific sera to be useful, it is mandatory that their composition be known. Absorption is still a tedious process and requires large numbers of cells. The differential distribution of the leukocyte antigens on the fixed tissues has been little studied. Large quantities of specific sera are needed for the determination of tissue concentration of antigen, for reliable family studies, and for standardization of reagents between laboratories. Chance segregation of antigens and genetic linkage prevent the substitution of family studies for careful absorption. The agglutinating activity of a monospecific serum should be completely removed by absorption with any of the cells with which it reacts, and then further checked for monospecificity in family studies.

Of the sources of sera we have investigated, the sera from multigravida appear to offer the richest variety. While many women appear to retain their antibodies for many years without obvious alteration in specificity, examples are well documented of a complete loss of activity or a marked change of specificity. Antibodies in the serum of immune chimpanzees, on the other hand, appear to remain stable. If Falweb is a typical example, it is to be anticipated that the antibodies will retain the same reactivity and approximately the same titer for extended periods, thus allowing one to build up a considerable stock of antibody. With adequate typing of the donor and antibody producer, either human or chimpanzee, it may be possible to produce specific antibodies experimentally. This would greatly ease the problem of obtaining standard reagents for use in any laboratory involved in tissue typing.

Summary

Sera have been obtained from three sources: from parous women, from volunteers immunized against lymphocyte preparations, and from chimpanzees immunized with suspensions of human buffy coat cells. The sera differ in their reactivity, but strong sera from each source appear to contain a mixture of antibodies with individual specificity. The antibodies have all been tested by a modified leukoagglutinin test using micro volumes, siliconized tubes, and leukocyte suspensions virtually free from platelets.

Occasional leukoagglutinins were found in sera from primigravida, but these sera together with many samples from multipara were of low titer and gave inconstant results. Sera of medium reactivity, with titers of about 1:8, may contain more than one antibody, and most strong sera contain a complex mixture of antibodies separable by repeated absorption with leukocytes.

Antibodies have been found in the serum of seven of nine subjects injected with lymphocyte suspensions. The effective immunizing dose has been as low as 5×10^7 cells. At present these antisera are of moderate titer and also contain a mixture of antibodies.

The chimpanzees immunized with buffy coat cells from individual human donors responded in several ways. Some animals failed to produce leukoagglutinins after prolonged immunization. Others produced antibodies to an antigen present on all human leukocytes. In addition, some of the chimpanzees also produced antibodies to human leukocyte isoantigens. These antibodies can be made monospecific by absorption techniques.

Sera from the various sources are being used to group human leukocytes. Many of the antibodies so far obtained are not monospecific reagents but can be used in conjunction with pure antibodies for grouping human subjects.

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Discussion

DR. van ROOD: I want to make one small comment on the immunization method. Dr. Amos pointed out that it is not always easy to get leukocyte agglutinins. For that reason I want to give you a method which, in a study performed with Dr. Balner and Dr. Cleton, has proved to be about 100-percent successful in man, dogs, and monkeys. When you inject leukocytes intravenously, leukocyte agglutinins are produced in about 25 percent of the recipients. I think this is in accordance with the data of the French workers, Dr. Payne, ourselves, and many others. If you inject the buffy coat of about 10 or 20 ml of whole blood intracutaneously once or twice, you will find homograft sensitivity, as has been pointed out by Dr. Merrill and Dr. Rapaport, but you rarely find leukocyte agglutinins. However, if you give one or two intracutaneous injections and 7 to 10 days later one or two intravenous injections from the same donor, you find both homograft sensitivity and, in our hands, about 100 percent leukocyte agglutinins.

That is the way we immunized dogs. In man and monkeys, rather than inject leukocytes intradermally, one or more skin grafts were transplanted. The data from humans were presented in more detail in New York at the VI International Transplantation Conference (February 1964). If I understand Dr. Rapaport correctly, there is—as an immunizing stimulus—not much difference between a skin graft and the intracutaneous injection of leukocytes. This is, in our opinion, the most reliable and practical way to make leukocyte agglutinins.

DR. HIRSCHHORN: How long do the agglutinins last?

DR. van ROOD: We have picked them up 2 years later.

DR. HIRSCHHORN: Did you titer them?

DR. van ROOD: We have had titers up to 1 to 128.

DR. HIRSCHHORN: A week between injections?

DR. van ROOD: From a week to 10 days. This is not important.

DR. SHULMAN: Concerning the development of leuko-complement fixing antibodies, at least, they have disappeared rather rapidly from most maternal sera within a period of several months, but they have lasted as long as 4 to 6 years. I don't see how you can predict in any one instance whether a particular antibody will disappear. On the other hand, we have been almost unable to produce anamnestic responses. This wasn't done purposefully, but in individuals who had titers that disappeared, who later received transfusions with cells containing the antigen, we have yet to see a reasonable anamnestic response with a single injection. Most of the individuals require multiple transfusions.

DR. van ROOD: Did you start off with intracutaneous injections?

DR. SHULMAN: These were all intravenous. These were transfusions.

DR. van ROOD: I think that the fact that the first was an intracutaneous injection might be important. Another point which I would like to make: I'm not completely convinced that the 25 percent positive that you find after an intravenous injection is not perhaps partly due to leakage from an intravenous injection intradermally.

DR. MILGROM: I think that the line of investigation selected by Dr. Amos and his associates is most promising. There existed a great deal of hesitation in using animal immune sera for distinguishing human isoantigens. This hesitation was created by what Landsteiner called "false perspective," meaning that an animal which is biologically distant from the species whose antigens are used for immunization cannot properly distinguish the subtle differences between these antigens. Interestingly enough, Landsteiner himself proved that there are important exceptions to the false perspective rule. Human MN blood groups were discovered by Landsteiner and Levine in using rabbit immune sera and I doubt whether these antigens would have ever been discovered if heteroimmune sera had not been applied.

I wonder why Amos *et al.* limited their investigation to primates. It would still be worthwhile to explore the possibility of using immune sera obtained in small laboratory animals, such as rabbits and guinea pigs, which might be capable of distinguishing some of the human leukocyte isoantigens. The second comment that I wanted to make concerns the use or misuse of the term "organ-specific antigens." Organ-specific antigens are defined as any other tissue. Most organ-specific antigens cross species lines and have a very similar or even identical serological structure in many species. Leukocytes can be considered an organ and we have reasons to believe that there exist organ-specific antigens in leukocytes. Recently in my laboratory, Dr. Colombani demonstrated that there are thermostable, ethanol-insoluble antigens which are just characteristic for leukocytes as a tissue.

Can an isoantigen be an organ-specific antigen? This may sometimes happen. Rose, Metzgar, and Witebsky (1960) described organ-specific antigens characteristic for rabbit pancreas which were also isoantigens. But I would call this situation rather unusual, since most isoantigens are not restricted to one organ. In this connection, I would not expect that leukocyte antigens which have been discussed here are really organ-specific antigens and that, sooner or later, they will be found in other tissues as well.

The third comment concerns Dr. van Rood's presentation. It would be most interesting to find out whether the immune reaction responsible for the graft rejection is directed against the same antigens as the humoral antibodies detected by test-tube procedures. Considering that delayed hypersensitivity reactions might play the most important role in homograft rejection, the question arises: Against what antigens are these reactions directed? This point certainly does not diminish the importance of detecting humoral antibodies which may indicate a serological incompatibility between the host and recipient.

DR. LAWRENCE: Earlier work with bacterial hypersensitivity, which showed, as Dr. van Rood pointed out, that the intravenous route in the guinea pig, using tubercle bacilli, streptococci and pneumococci, will not induce delayed allergy in the animal, although it will induce an immune response

usually in the serum. This was also shown later by Medawar and subsequently by others, that if one uses the intravenous route for skin homograft reaction, you were apt to desensitize, or prolong, graft survival rather than cause accelerated rejection. And I would add to Dr. Milgrom's comment that in this instance we haven't yet really come to grips with the mechanism or mechanisms involved in homograft rejection. The data which Dr. van Rood presented would suggest again that the acquisition of delayed allergy, whether or not accompanied by serum antibody, might be the important event for which we are really looking.

DR. WILSON: The volunteers were arranged in donor pairs on the basis of erythrocyte (ABO) compatibility and leukocyte incompatibility as reflected in the agglutination reactions of their leukocytes with a panel of leukocyte-agglutinating sera of patients who had received multiple transfusions. (Table 1)

Table 2 indicates the amount of antigen injected into each recipient and the time required for the development of leukocyte antibodies. For example, in recipient I, antibody appeared in 111 days and in his partner (number IX), 249 days. As you see, the development of antibodies varied from 40 to 293 days from the initial injection and the total quantity of antigen required varied from 1.74×10^9 to 13.5×10^9 cells. These agglutinins were demonstrable in the blood for a period varying from 72 to 223 days. In no case did antibodies reappear after they had disappeared. To our knowledge, there has been no indication of autoimmune disease in any of these volunteers.

DR. van ROOD: Would you like to answer exactly to the route of immunization?

DR. WILSON: The initial injection was mixed with mannide-oleate (Arlecel-A) adjuvant; one half was given intradermally, the other half intramuscularly. Subsequent antigens consisted of saline suspensions of twice-washed leukocytes without adjuvant. Several of the injections after the initial one were divided equally between intradermal and intramuscular routes. The final antigen injections were intramuscular. Four of the ten subjects received part of the antigen intravenously in the form of cross-transfusions. Two of these volunteers received most of the antigen intravenously; it proved to be comparatively unproductive. A comparatively large quantity of antigen was required to induce antibodies by this route.

DR. BRENT: At the recent transplantation conference in New York, Dr. van Rood gave some figures which were an attempt to correlate his leukocyte typing with skin-graft compatibility or incompatibility. If I remember correctly, the difference between the compatible and incompatible groups was on the order of 2½ days, which Dr. van Rood, at that time, considered not to be

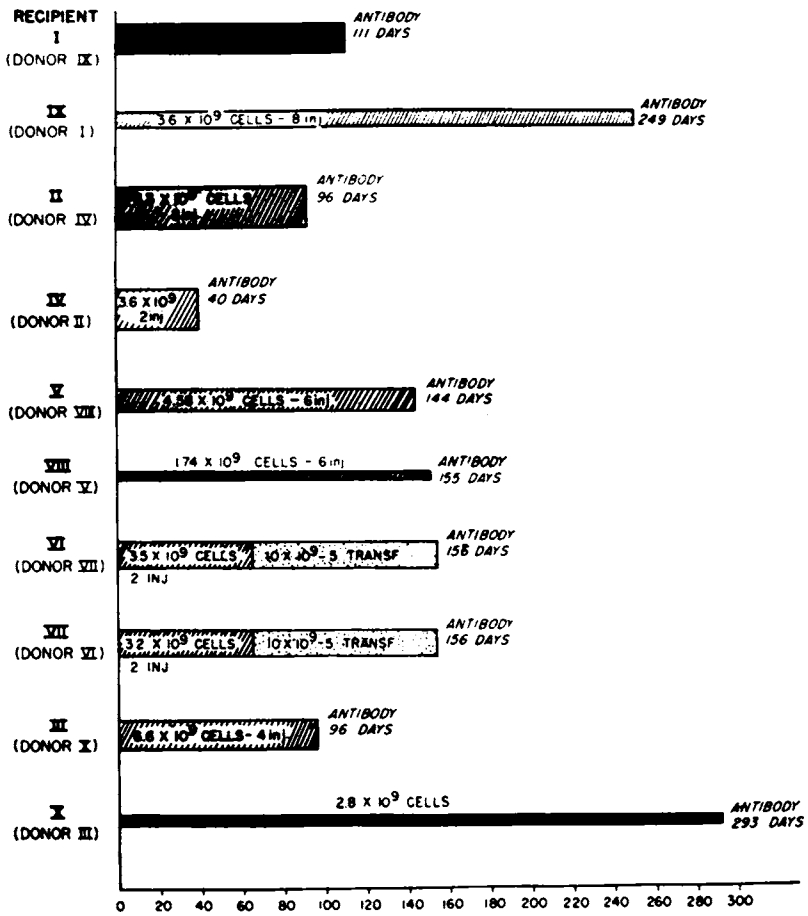
TABLE 1

MATCHING OF DONOR PAIRS ON BASIS OF ANTIGENIC SPECIFICITY OF LEUKOCYTES (AGGLUTINATION)

Leukocyte Donor		Antisera							Erythrocyte Type	
		1188	1523	2338	2609	1785	2399	NORMAL Serum	ABO	Rh
COR	(1)	-	+	+	+	+	–	-	A	+
McNE	(9)	+	+	+	-	–	–	–	A	–
DUN	(7)	–	+	+	+	+	+	–	O	+
BEAV	(6)	–	+	+	–	-	-	–	O	+
SCHMA	(5)	- -	–	–	–	+	–	–	O	+
KEL	(8)	-	+	+	+	+	-	–	O	–
STRI	(4)	-	+	+	+	+	+	–	B	+
FRE	(2)	–	+	+	–	+	–	–	B	+
BRO	(3)	–	–	–	+	+	–	–	A	+
SCHRAM	(10)	-	+	+	+	+	+	–	A	+

TABLE 2

DEVELOPMENT OF LEUKOCYTE ANTIBODY



significant. I wonder, since he's obviously made great advances since February, whether he has any further information and whether he has made any further attempts to produce such correlations.

DR. van ROOD: The answer is no. The point I wanted to bring out in New York was that, if you do typing with sera of which you are not certain that they may recognize one or more antigens, then your typing and your matching will be unreliable. Furthermore, I considered the difference of 2½ days as not significant because we had too little experience in grafting.

DR. DAUSSET: I should like to say that with Dr. Colombani we have carried out a similar experiment. Using twelve normal recipients we obtained differences of less than 2 days, the correlation seeming to be doubtful: we used six Hodgkin's patients as recipients and found longer differences. This work was also presented in February at the VI International Transplantation Conference.

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Complement Fixation in Typing Histocompatibility Systems*

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The capacity of complement to combine with a wide variety of antigen-antibody complexes has served as a basis for the development of serological tests that have been employed as diagnostic and experimental tools for more than 60 years. During this time, and particularly in recent years, the general nature and mechanisms of such tests have been the object of intensive study and a number of excellent reviews have appeared (Rapport and Graf, 1957; Osler, 1958, 1961; Mayer, 1961). General technical descriptions of the tests can be found in virtually any text on immunology and the matter is treated exhaustively by Mayer (1961). Accordingly, it seems appropriate to consider, at least briefly, the necessity for discussing here some technical problems associated with the application of complement fixation to the study of isoantigens.

Although it is virtually impossible at the present time to carry out precise quantitative studies on most tissue antigens, there are good reasons for believing that the antigens which distinguish between animals within a species are present in cells in much smaller amounts than those antigens which are commonly described as species specific. Moreover, cellular isoantigens have only occasionally been obtained in a soluble form, so that the study of isoimmune reactions has been for the most part an investigation of the interaction of relatively dilute solutions of antibody molecules with cells or subcellular particles containing low concentrations of antigens. It is not surprising, therefore, that investigators in this field have often found it necessary to modify many of the standard serologic procedures in order to develop a reliable methodology. The low concentration of antigenic receptors on cell surfaces tends in general to minimize the extent of specific aggregation of such cells. While in some isoimmune systems agglutination may be observed to occur routinely in ordinary saline media, its occurrence in many systems depends on the presence of substances which promote aggregation, such as dextran, PVP, or high protein concentrations. Insofar as reliable agglutinating or cytotoxic procedures can be developed by such modifications of existing techniques there is little reason to introduce more demanding or technically complex tests such as complement fixation (CF) for routine typing, though the use of quantitative CF can, as illustrated by the work of Shulman, serve as a valuable tool in studying other aspects of isoimmunization.

There are, however, many isoimmune systems whose existence is indicated by transplantation studies, but for which no adequate methods of serological analysis are available. This may in some cases reflect the inadequacy with which the systems have been investigated, but in other cases it is becoming increasingly evident that we are dealing with substances which are present in minute concentrations on cell surfaces and which are capable of eliciting only feeble antibody responses. Interaction of the antigens and antibodies involved in these systems does not result in readily detectable alteration in the state of the system, and it is necessary to resort to indirect methods of determining whether or not an immune reaction has taken place. It is in conjunction with these "weak" systems that the special properties of complement may be expected to find some important application to tissue typing.

There are, unfortunately, some unusual problems that arise in connection with attempts to develop CF tests for these weak systems. Recent studies have emphasized the importance, in binding complement, of both the total number of antigenic receptors per cell and the distribution of these sites on the surface of the cell. The relatively sparse distribution of isoantigenic receptors tends to restrict the participation of comple-

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ment in isoimmune reactions and there may, indeed, be a number of systems in which the geometry of the receptors is entirely unfavorable for the development of the specific interactions which initiate the fixation of complement. Additional difficulties center about the interference which is characteristic of both the antisera and the antigenic preparations. In order to supply a sufficient amount of antigen in these weak systems it is necessary to introduce a disproportionately large amount of nonreactive cellular material which often inactivates a considerable amount of complement, and the antisera must be employed at concentrations that may strongly inhibit or strongly enhance the activity of the test complement. This over-all interference poses serious technical problems in the elaboration of suitable CF techniques and it may be properly viewed as the principal limitation to a more widespread use of CF tests in tissue typing.

Our own interest in applying CF techniques to isoimmune systems stems originally from attempts to employ quantitative serological procedures in an investigation of a variety of genetic and immunological problems associated with homotransplantation of tissues in mice. For these early studies sera were selected on the basis of their high reactivity in hemolytic and cytotoxic tests. Our recent efforts to extend these studies to less potent sera and to non-H-2 systems must be regarded as essentially preliminary in nature. There are in this area of research no standard or widely accepted procedures and it would be impossible to use the presently available techniques in any but the most crude type of immunogenetic analysis. It seems worthwhile, however, to describe those techniques that have met with some success, to indicate existing difficulties and pitfalls in these techniques, and, where possible, to point in the direction along which future developments may be expected to take place.

While it is unnecessary to engage here in any discussion of the basic principals of CF, there are some technical aspects of the procedure which require some special consideration in connection with its application to the study of cellular isoantigens. Since relatively high concentrations of complement must be employed to offset the interference introduced by the particular type of immune system that is being analyzed, it is imperative that the complement be free of normal antibodies capable of reacting with the test tissues. Indeed, with very weak systems the levels of such antibodies may exceed those present in the immune sera. Accordingly, all complement preparations used in the present study were thoroughly absorbed at low temperatures with mouse tissues.

It may at times be desirable to employ complement from species other than guinea pigs, and in some situations there may be a decided advantage in utilizing the complement of the very animal that is being typed. In any case it is important to select an appropriate indicator system, since we have shown, for example, that while guinea pig complement is more efficiently activated by 19S antibodies for sheep erythrocytes than by the lower-molecular-weight antibodies, the reverse is true for mouse complement and rabbit complement, and we have found that the use of the proper type of hemolysin greatly increases the sensitivity of tests carried out with these complements.

Detailed description of the techniques that we have employed will be omitted here except insofar as they represent some unusual departure from the procedures described by Mayer (1961).

Figure 1, which is a modification of previously published material, contains the results of a quantitative CF test in which various numbers of strain A/Sn lymphoid cells were incubated at 37°C for 90 min with a fixed concentration (1:160) of antiserum and guinea pig serum containing $50C'H_{50}$. This was a relatively potent antiserum that could be used at a dilution of 1:160, a concentration that is fortunately only slightly anticomplementary. Moreover, it was known to contain antibodies to several individual components of the H-2 system and this presumably accounts for the fixation of significant amounts of complement, even with low cell concentrations. In the case of lymph-node cells the degree of fixation rises sharply with increasing cell concentration, to an optimal value after which further increases in the amounts of antigen result in a decline in the amount of complement fixed. The absence of an optimal degree of complement fixation with thymus cells reflects the difficulty of attaining antigen excess with these cells, which had been shown to contain very low concentrations of the isoantigen involved in this system. These data were collected in connection with studies quite unrelated to the topic we are considering here today, but they do illustrate quite clearly the feasibility of detecting H-2 antigens by means of CF, a point that was originally established by Batchelor (1960). It is particularly perti-

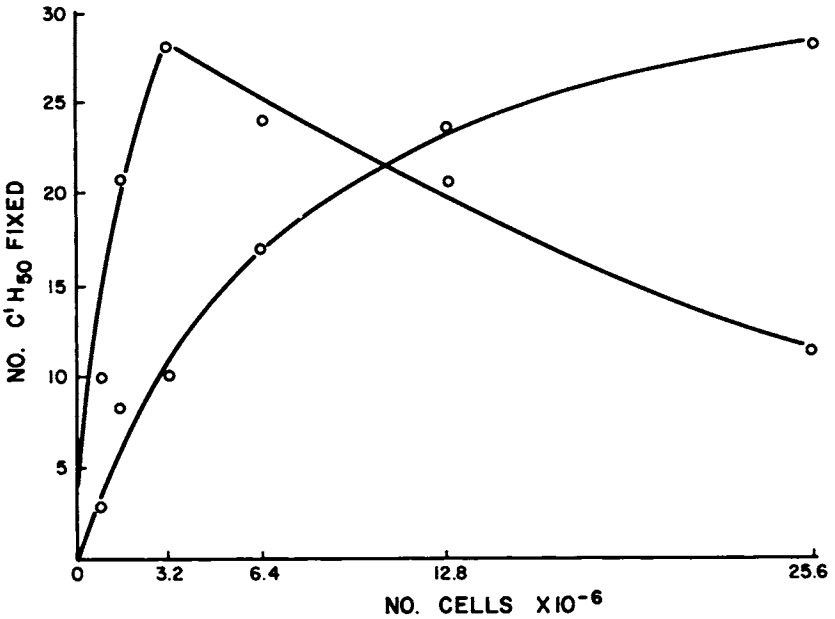


Figure 1.—Fixation of guinea pig complement by A/Sn lymphoid cells and C57BL/10 anti-A/Sn serum (1:160). o———o thymus cells; •———• lymph-node cells.

nent to note that thymus cells, which were neither agglutinated nor killed by the antiserum employed in this test, served satisfactorily as CF antigens.

Data similar to these can be obtained with a variety of H-2 antisera and the development of a simple qualitative test presents no problem with these relatively strong systems. There are, however, numerous cases in which the fixation of complement is observed only when large numbers of cells are allowed to react with relatively high concentrations of serum, a combination of circumstances which often leads to excessive interference. The effect of fresh normal mouse serum on the hemolytic activities of guinea pig and rabbit complements is shown in Fig. 2, which illustrates the results of a series of complement titrations carried out in the presence of various concentrations of mouse serum. The activity of guinea pig complement is markedly depressed by the higher concentration of mouse serum, but, even when the latter was employed at a dilution of 1:160, some inhibition could be readily detected. Rabbit complement activity, on the other hand, was significantly enhanced by mouse serum, though the degree of enhancement was diminished at higher serum concentrations, suggesting the operation of inhibitors even in this system.*

This type of interference can be reduced considerably by heating mouse serum at 56°C for 20 to 30 min, though it still remains at a level that cannot be ignored in quantitative tests. We have, accordingly, modified our quantitative CF procedure for use with weaker antisera by mixing the tissue cells with the antiserum, allowing a suitable period of incubation, sedimenting the cells by centrifugation washing, and resuspending them prior to the addition of complement. The results of a test carried out in this manner with an H-2 serum of low reactivity are shown in Fig. 3. Relatively high concentrations of cells were required to demonstrate significant fixation of complement and considerable inactivation was observed with cells that had been inoculated in normal serum. The latter could, however, be easily distinguished from cells that had been allowed to react with immune

* It should be emphasized that the enhancing and inhibitory properties of mouse serum described here were observed in conjunction with sheep erythrocytes that had been sensitized with 19S hemolysins. When 7S hemolysins are employed, mouse serum interferes with the titration of guinea pig complement in a different manner (Winn, 1964).

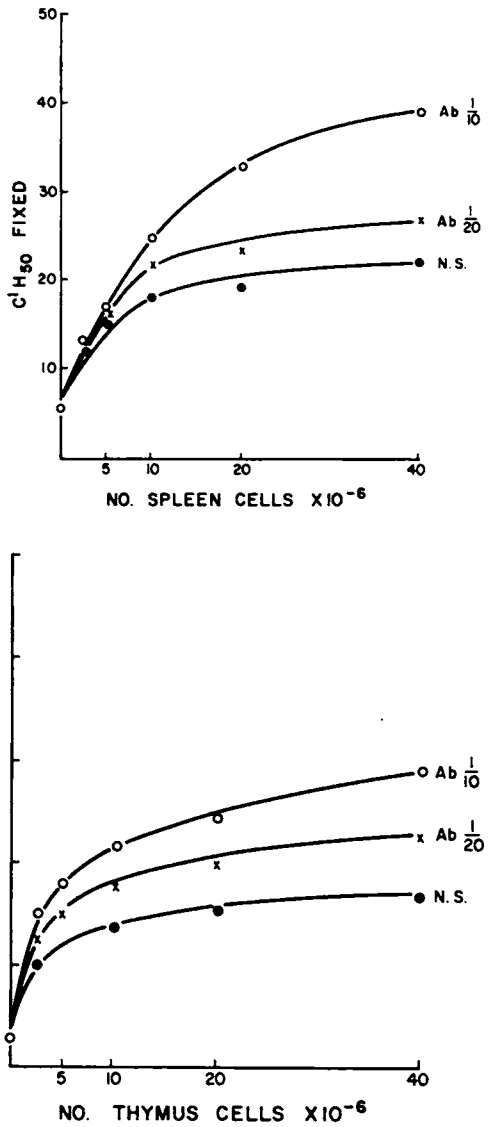


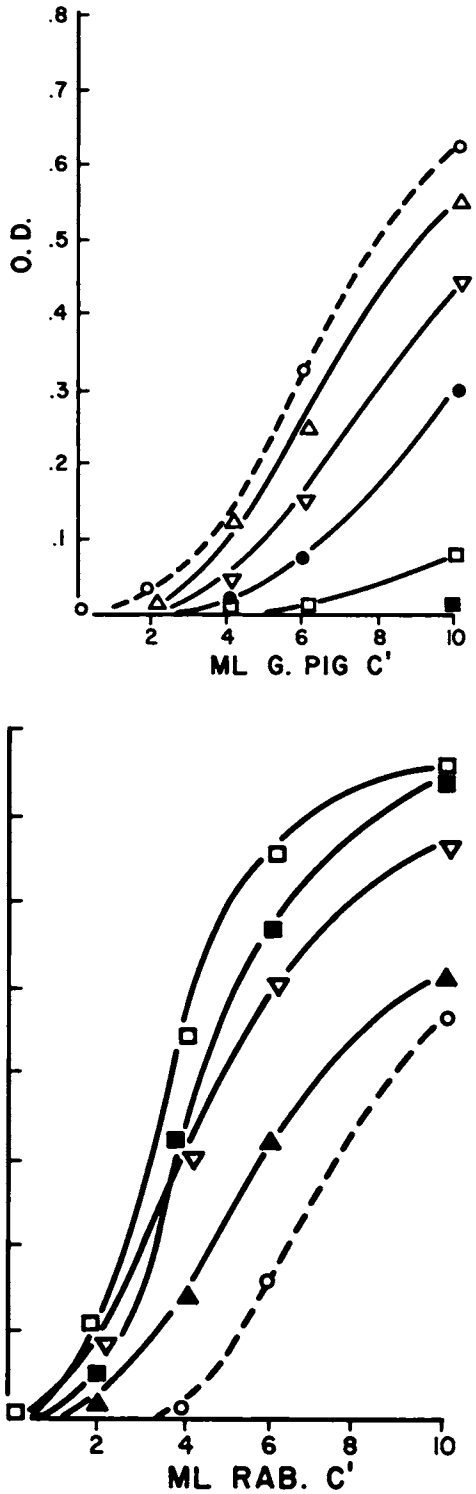
Figure 2.—Fixation of guinea pig complement by A/Sn lymphoid cells and A.SW anti-A/Sn serum or normal serum. Sera were removed from cells prior to the addition of complement.

serum, and cells from mice of the same strain as the antiserum donors did not show immune fixation, thus establishing the specificity of the test. Thymus cells were again found to be less reactive than lymph-node or spleen cells, and additional tests showed that cells from mice that were heterozygous for the genes determining the specificities of the antigens involved in these reactions were much less reactive than those from homozygous animals.

Fortunately, the very high anticomplementary activity of mouse serum is exceptional and is not encountered in samples of normal human serum. However, sera from some patients may exhibit anticomplementary properties and such activity may be acquired by normal serum on prolonged storage. It is imperative, therefore, in all CF tests that inactivation of complement by serum alone be eliminated or accounted for quantitatively. The tendency of sera, especially in high concentrations, to enhance the hemolytic activity of complement can usually be satisfactorily reduced by heating at 56°C for 20 to 30 min, a procedure which, as stated above, also diminishes anticomplementary effects.

The inactivation of complement by cells mixed with saline and normal serum is by no means restricted to mouse tissues and this source of interference must be strictly controlled in all tests. The explanation for this type of interference is not at all clear, though the possibility exists that complement contains small amounts of normal antibodies that are not easy to remove by absorption with tissue for short periods of time at low temperatures. This explanation receives some support from data, to be reported below, that with some lots of complement only slight inactivation is observed with unsensitized cells. Nevertheless, it is a commonly encountered problem which is often obscured by the design of some qualitative tests.

Quantitative CF tests serve a number of useful purposes in laboratory experiments and they may very well prove of considerable value in general typing problems, such as distinguishing between individuals that are homozygous or heterozygous for genes determining isoantigenic specificity. They are, however, obviously unsuitable for wide-scale typing procedure, and we have discussed them here largely because they illustrate some important aspects of CF reactions which are not at all apparent in qualitative tests. We have, moreover, used the data obtained in such tests as a basis for the elaboration of some modifications of routine diagnostic procedures which make proper allowance for the nonspecific inactivation inherent in the systems which we have been discussing. Prime consideration has been given to the development of techniques that will permit the determination of an animal's phenotype in terms of appro-



priate test sera with an extremely high degree of certainty, and technical simplicity has, at times, been sacrificed in favor of reproducibility and hence reliability of test procedures.

Initially, we studied the role of the following variables in CF tests: (1) species of complement; (2) time and temperature of incubating the various reactants; (3) the concentration of antigen and antibody; and (4) the concentration of complement. A rather large number of tests were carried out to determine the optimal conditions for fixation, but these need not be presented in detail here, and the results will instead be summarized briefly.

Species of complement. Complement from mice, rabbits, and guinea pigs was investigated. Mouse complement showed very little nonspecific fixation with mouse tissues, but had extremely low lytic activity which deteriorated spontaneously during incubation. Rabbit sera were found to have considerably higher levels of complement but even after several absorptions at 0°C with mouse tissues, there was a high degree of inactivation of this complement by unsensitized mouse cells. We have, accordingly, employed guinea pig complement in all of the tests to be reported here.

Time and temperature of incubation. Tests carried out at 0 to 4°C for 18 to 24 hr were accompanied by some spontaneous loss of complement in controls and by considerable amounts of fixation by cells and normal serum. Tests incubated at 37°C were found to be at least as sensitive as those carried out in the cold and they were invariably more reliable. A period of 90 min resulted in maximal fixation with negligible spontaneous loss of lytic activity in controls.

Concentration of antigen and antibody. The optimal concentrations of these reagents vary with the particular system being

Figure 3.—Titration of guinea pig complement (1:100) and rabbit complement (1:10) in the presence of fresh normal mouse serum. Sheep erythrocytes were sensitized with a commercial preparation of rabbit hemolysin containing predominantly 19S antibodies.

o——o control titration in absence of mouse serum;

△——△ mouse serum 1:160; ▽——▽ mouse serum 1:80;

●——● mouse serum 1:40; □——□ mouse serum 1:20;

■——■ mouse serum 1:10.

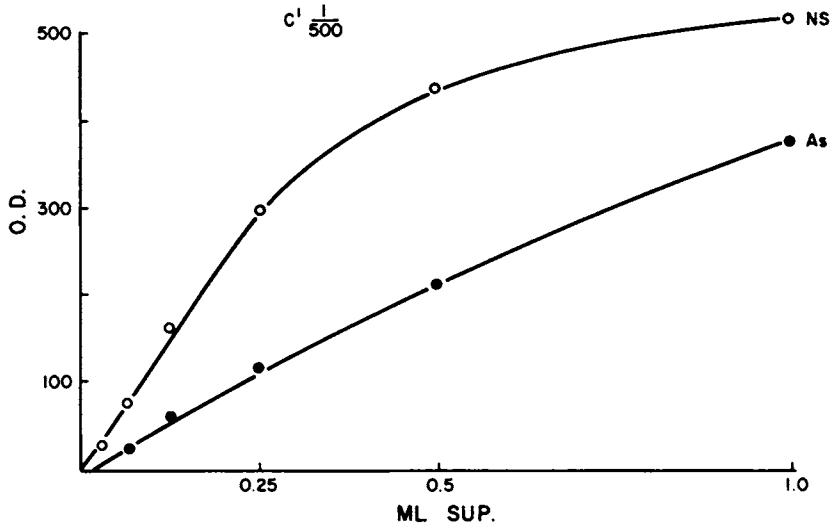


Figure 4.—Titration of mouse C'1 following incubation with C3H/Sn lymphoid cells previously treated with normal serum or C3H.K anti-C3H/Sn serum.

studied and it is strongly urged that all CF typing tests be carried out with more than one level of antigen. The range of concentrations which we have found to be appropriate will become evident in the presentation of our data.

Concentration of complement. We have employed amounts of complement varying from 2.5 to 40 $C'H_{50}$. Following incubation of sensitized mouse cells with complement the mixtures were centrifuged and the supernatant fluid was titrated for hemolytic activity using sensitized sheep erythrocytes. A semiquantitative procedure was employed in which doubling dilutions of the supernatant fluid (0.2 ml) were mixed with an equal volume of the indicator system, incubated for 60 min at 37°C, and read visually for lysis. With high concentrations of complement there is an obvious lack of sensitivity, since modest degrees of fixation do not produce sufficiently marked differences between test and control titration. Very low concentrations of complement result in a high degree of sensitivity but this is, unfortunately, often achieved at the expense of reliability. We have found that systems containing 5 to 10 $C'H_{50}$ provide, with our experimental materials, a reliable and reasonably sensitive test.

Table 1 shows the results of a CF test involving a relatively strong antiserum prepared in C57BL/10 mice that had received a series of injections of A/Sn lymphoid tissue. This serum, which was heated at 56°C for 20 min, displayed some anticomplementary activity but was obviously quite suitable for the detection of appropriate isoantigens. Ten $C'H_{50}$ were employed in this test and it can be seen that a substantial amount of the complement was inactivated by C57BL/10 cells in the presence of antiserum. Only slight inactivation was observed when similar cells were incubated with complement alone or with complement plus normal serum. No explanation can be offered for this apparently nonspecific fixation which is observed rather commonly with mouse antisera. There is, however, the possibility that some hyperimmune sera contain autoantibodies or cytophilic antibodies that are capable of fixing complement even when bound nonspecifically. In any case, this type of inactivation of complement could, with the serum under consideration, be distinguished easily from specific immune fixation as shown in Table 1. With this antiserum it was possible to demonstrate specific immune reactions with fresh tissue homogenates and with homogenates that had been lyophilized and stored for several years.

With weaker H-2 antisera it was found necessary to increase the sensitivity of CF tests by reducing the amount of complement employed in the system to 5 $C'H_{50}$. With this relatively low concentration of complement, the anticomplementary activity of heated mouse serum may obscure weak reactions and we have modified the procedure by re-

TABLE 1

CF with C57BL/10 anti-A/Sn Serum

Supernatant dilution	Antiserum Dilution									C^1 Control
	1:10			1:40			1:160			
	I	II	III	I	II	III	I	II	III	
1:1	0	4	C	0	C	C	C	C	C	C
1:2	0	3	C	0	C	C	4	C	C	C
1:4	0	1	4	0	3	4	1	4	4	C
1:8	0	0	1	0	1	1	0	2	3	3
1:16	0	0	0	0	0	0	0	0	1	1

C — complete lysis; 0 — no lysis; 4, 3, 2, 1 — intermediate degrees of lysis.
I = A/Sn cells; II = C57BL/10 cells; III = antiserum plus complement with no cells.

moving the antiserum prior to addition of complement. The results of a CF test in which this modification was used are shown in Table 2. In this particular test, cells that had been incubated with normal serum inactivated only trace amounts of complement and specific immune fixation could be detected with cell concentrations as low as 2.5 to 5 x 10^4/ml, using antiserum at a dilution of 1:120. A.SW lymphoid cells that had been incubated with the A.SW anti-A/Sn serum could in every case be distinguished from A/Sn cells that had been treated similarly.

Attempts to apply these typing procedures to the detection of non-H-2 antigens have not been successful. In several cases the amounts of fixation were surprisingly high but the specificity of the reactions could not be established.

Table 3 shows the results of a CF test (5 $C'H_{50}$) in which C57BL/10 lymphoid cells and 129/ lymphoid cells were mixed with either normal serum or a C57BL/10 anti-129/ serum. These two strains of mice have been classified as H-2B so that the antiserum employed here would be expected to contain no antibodies specific for H-2 antigens. It can be seen from the data that neither type of cell inactivated complement after incubation with normal serum. There was, on the other hand, marked inactivation of complement by both cell types following incubation with immune serum. Again no explanation for this apparently nonspecific fixation can be offered and can find no reason for finding such high reactivity of this type in antisera of this kind. Ten different samples of normal serum failed to disclose any ability to make cells anticomplementary and eight samples of antiserum of the general type employed here showed this strong nonspecific reactivity. The presence of autoantibodies or cytophilic antibodies may, as stated previously, be in part responsible for such reactions. We are now attempting to prepare antisera to non-H-2

TABLE 2

CF with A.SW anti-A/Sn Serum and A/Sn Cells

Supernatant dilution	Antiserum Dilution																C^1 Control
	1:30				1:60				1:120				normal serum (1:60)				
	A	B	D	E	A	B	D	E	A	B	D	E	A	B	D	E	
1:1	0	1	1	C	0	1	4	C	1	1	C	C	C	C	C	C	C
1:2	0	0	0	4	0	0	1	4	0	1	1	C	C	C	C	C	C
1:4	0	0	0	1	0	0	0	1	0	0	1	1	4	4	4	4	4
1:8	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	1	2

C — complete lysis; 0 — no lysis; 4, 3, 2, 1 — intermediate degrees of lysis.
A = 20 x 10^6 cells; B = 10 x 10^6 cells; D = 5 x 10^6 cells; E = 2.5 x 10^6 cells.

TABLE 3

CF with C57BL/10 anti-129/ Serum

Supernatant dilution	C57BL/6 cells								129/Cells								C^1 Control
	Norm. Serum				Antiserum				Norm. Serum				Antiserum				
	A	B	D	E	A	B	D	E	A	B	D	E	A	B	D	E	
1:1	C	C	C	C	0	1	2	C	C	C	C	C	0	1	1	2	C
1:2	4	4	4	4	0	0	1	2	3	4	4	4	0	0	0	1	4
1:4	1	1	1	1	0	0	0	1	1	1	1	1	0	0	0	1	2

C — complete lysis; 0 — no lysis; 4, 3, 2, 1 — intermediate degrees of lysis.
A = 40 × 10^6 cells; B = 20 × 10^6 cells; D = 10 × 10^6 cells; E = 5 × 10^6 cells. Both normal and immune sera were used at a dilution of 1:10 and the sera were removed from the cells prior to addition of complement.

isoantigens in other strain combinations, since it is possible that what we have observed here is peculiar to this experimental model.

Finally, we have attempted to devise a typing test based on the fixation of a single component of complement. Recently (Winn, 1964) we have shown that the action of guinea pig complement on sheep cells sensitized with 7S hemolysins can be strikingly enhanced by the presence of mouse C'1 which is, fortunately for our purposes, the first component which becomes fixed by antigen-antibody complexes. We have taken advantage of this phenomenon in developing the following procedure. Mouse cells are mixed with isoimmune serum and small amounts of fresh mouse serum, and the mixture is incubated at 37°C for 15 to 30 min. The cells are then separated from the supernatant fluid which is titrated for residual mouse C'1 activity. The latter titration is carried out by adding varying amounts of the supernatant to a suspension of sheep erythrocytes that are sensitized with 7S hemolysins. A sublytic dose of guinea pig complement is then added and the tubes are incubated at 37°C for 60 min. The degree of hemolysis is then determined colorimetrically. It would be inappropriate to discuss these reactions here in greater detail, but some indication of the types of results we have obtained will be summarized.

Reactions involving H-2 antigens can be readily detected, since these systems may fix as much as 100 units of C'1.* We have applied this technique to only one antiserum that contained no H-2 antibodies. This serum was prepared by injecting C3H/Sn tissues into C3H.K mice; these strains differ only at the H-1 locus. C3H lymphoid cells were incubated with either normal or immune serum in the presence of fresh mouse serum diluted 1:500. The results obtained by titration of the supernatant fluids are presented graphically in Fig. 4. Nonspecific fixation was not observed with this serum, which appears to contain only very small amounts of antibody. The technique is, of course, technically elaborate when compared with other typing procedures but it has permitted in this instance the detection of specific reactivity in a serum which was nonreactive in all other serological tests.

The results of all of these CF procedures may be summarized as follows:

(1) Strong H-2 systems fix substantial amounts of guinea pig complement and these systems may be studied by both quantitative and qualitative procedures. The antigens may be in the form of intact cells or of tissue homogenates that have been freshly prepared or stored in either the frozen or lyophilized state.

(2) Weak H-2 systems are not suitable for quantitative CF studies but qualitative tests can be devised for typing the antigens involved in such systems.

(3) Some non-H-2 systems definitely fix complement but the problem of specificity in these systems remains to be solved.

* One unit is defined as the amount of fresh normal mouse serum which will produce 50-percent lysis of sensitized sheep cells in the presence of an amount of guinea pig complement that by itself will lyse between 0 and 5 percent of such cells; most samples of mouse serum contain between 2,000 and 10,000 units as defined here.

(4) The use of procedures which detect the fixation of C'1 offers some promise in developing typing tests for very weak histocompatibility systems.

The development of CF typing tests for species other than the mouse may proceed without involvement of many of the technical problems that have been considered here, but it is unlikely that standard diagnostic tests can be used unmodified in the analysis of many histocompatibility systems. It is particularly important that investigations in this area of research consider the use of a variety of sources of complement and their appropriate indicator systems.

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Discussion

DR. SHULMAN: If I had known there were so many pitfalls, I never would have undertaken complement fixation. With human antibodies and human or guinea pig complement, the anticomplementary problems discussed by Dr. Winn do not arise. All the human platelet and leukocyte isoantibodies that we have seen are 7S gamma globulin, and all fix a little more guinea pig complement than human complement. In contrast to mouse sera, human sera, even after storage for long periods, rarely show more than one unit of anticomplement activity at concentrations used in complement-fixation tests.

I think one might get the impression from the use of mouse antibodies to study the H-2 locus that complement fixation is an inordinately complex technique, not only because anticomplementary effects occur, but also because reactions apparently are not typical of monospecific antibodies. For instance, Dr. Winn's curves with increasing cellular concentration show an asymptotic value rather than an optimum. This finding is characteristic of antisera containing antibodies against several antigens, each with a different cellular optimum.

DR. WINN: The anticomplementary activity of mouse serum is well documented in the literature and I do not think that there is any need to discuss the point in detail at this time. I know that a number of people at this meeting have observed "prozones" in cytotoxic tests when they have used mouse antisera in conjunction with guinea pig complement but not with rabbit complement, and I think that the data which I have shown here explain this very nicely.

I should like also to comment on the relationship between the physical-chemical properties of antibodies and their abilities to fix complement. The antisera that we have reported on here had a preponderance of 7S antibodies as judged by lack of sensitivity to 2-mercaptoethanol and by their behavior on columns of Sephadex G-200 and DEAE-cellulose. These sera, as you saw, were used in conjunction with guinea pig complement, which they fixed rather efficiently. However, I think a distinction must be made between the fixation of complement and the activation of complement to produce lysis. Certainly the lysis of sheep erythrocytes by guinea pig complement is mediated much more efficiently by rabbit antibodies of the 19S type than by the lower-molecular-weight antibodies, but I think it is pretty well agreed that 7S antibodies are more active in fixing guinea pig complement, and there may, indeed, be some relationship between the ability of these smaller molecules to fix complement and their inefficiency in providing immune hemolysis.

There are in our observations no suggestions that the species in which antisera are prepared play an important role in determining the kinds of complement which are fixed most efficiently; the most crucial factor appears to be the physical-chemical properties of the antibody molecules.

DR. BOYSE: My colleague, Dr. Stück, has recently found that the factor in mouse serum which is anticomplementary for guinea pig serum is destroyed by heating to 56°C,

so that most of the prozone of the cytotoxic test is removed by heating the antibody to 56°C. Normal mouse sera that has been heated to 56°C is no longer anticomplementary.

I should like to make one other point, in reference to a previous discussion, and that is that the TL antigen which Dr. Old and I described, in mouse thymus, is an example of an isoantigen that is strictly confined to one organ, the thymus. TL antibody is not absorbed out by any other tissue either *in vitro* or *in vivo*. It is strictly confined to the thymus, yet it is also an isoantigen because it is present in some mouse strains and not in others.

Finally, I should like to ask Dr. Winn about the absorption of toxicity from rabbit sera. We've made one or two odd observations here and I wonder if you can help us with them. The first is that absorption with mouse tissue was virtually ineffective at 0°C so that we had to go to higher temperatures to get any absorption by mouse tissues. The second point is that for some unexplained reason absorption was, to some extent, specific for the particular tissue used for absorption. In other words, if we absorbed with a particular ascites tumor, toxicity would be taken out preferentially for that tumor rather than another, and so on, suggesting perhaps heterogeneity among the antibodies.

DR. WINN: I would say that your results with rabbit serum are very much in keeping with what we have found. After extensive absorption of this type of serum with mouse tissues at 0°C we observed significant fixation of complement by cells mixed with normal serum or saline. We have called this "nonspecific fixation," but it may, indeed, have been due to normal antibodies which we were unable to remove.

DR. BOYSE: The real point of my question is whether you can really absorb out the toxicity of rabbit serum for mouse cells. If so, will you tell us how?

DR. WINN: We have never been successful in carrying out satisfactory absorptions of rabbit complement, and that is why we have not employed this type of complement in the tests reported on here.

DR. LAWRENCE: This is a very exciting control observation which seems to be getting into everybody's way; that of finding complement fixation in normal sera. Would you think, Dr. Winn, that this could represent the normal individual's synthesis of a serum antibody to his own degraded tissue constituents in progress all of the time? Or could it represent coating of his tissue cells with other antigens, bacteria, *E. coli*, or something else? As histocompatibility typing progresses this becomes an important point to think about in all of this testing, since we don't live in a germ-free world and since we also degrade, alter, phagocytize, and digest our own so-called normal cell constituents from birth onwards. The great assumption is made that the detection of serum antibody denotes an aberration when rather it may be that serum antibody to a tissue constituent could be the normal state of affairs that protects us against auto-immunity or homograft rejection.

DR. WINN: I think that the design and nature of our test system may very well permit detection of reactions of the type you have mentioned, Dr. Lawrence, and I am happy that you have brought up this issue because it gives us an opportunity to make note of the early work of Kidd and Friedwald, who demonstrated complement-fixing antibodies to normal tissues in the sera of the tissue donors. Certainly, this sort of thing would be expected to interfere with the detection of weak histocompatibility systems.

DR. MILGROM: Several years ago we were interested in the application of the complement-fixation test for detecting leukocyte isoantigens and isoantibodies (Milgrom *et al.*, 1957). This method is rather difficult, since the complement-fixation test requires strong antibodies, and leukocyte isoantibodies are rather weak. In addition, the complement-fixation test is a rather poor procedure for detecting insoluble tissue antigens, which usually are anticomplementary themselves. We tried to overcome this latter difficulty by preparing leukocyte isoantigens in a saline extractable form, which proved to be quite a promising procedure. I still feel that in using leukocyte suspensions as complement-fixing antigens, one always will encounter numerous difficulties.

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Characteristics and Evolution of Leukoagglutinins Developed in a Patient Receiving Multiple Transfusions from Selected Donors*

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1. Introduction

It is well established in man (Ceppellini *et al.*, in preparation; Friedman *et al.*, 1961; Rapaport, Lawrence, and Converse, in press) as well as in some experimental animals (Medawar, 1946; Volante *et al.*, 1962), that injection of leukocytes from a prospective donor enhances the reactivity of the recipient against a subsequent graft from that donor. Thus at least some histocompatibility factors are present on the white cells. It is thus justified to investigate whether leukocyte antigens, which are detected by serological reagents, have any relevance for the homotransplantation reaction. Because the two classes of antigens may be independent, the answer to this important question must be obtained experimentally by testing for each discrete leukoantigen. An antigen's presence in both donor and recipient will give some measure of histocompatibility as indicated by the survival time of a first skin graft or possibly the degree of "blast transformation" in mixed lymphocyte cultures (Bain, Lowenstein, and MacLean, this conference). This problem has been discussed previously (Ceppellini *et al.*, in press).

For such experiments the reliability of the serological tests, however, is of primary importance. In particular, the use of sera containing antibodies against more than one antigen can simulate a nonexistent compatibility between donor and recipient, because their leukocytes are agglutinated by different antibodies. Van Rood (1962) has called attention to still another source of error, namely, frequent cases in which the leukocytes absorb the antibody but are not agglutinated by it. As will be shown later, this phenomenon probably is related to the so-called "nonreproducibility" of agglutination and is due to some peculiarities of both the cells and the sera.

To obtain leukocyte typing of a high quality, we have undertaken an extensive project of immunizing suitable human subjects with white cells obtained from a single donor for each recipient. The general plan of the experiment has been described elsewhere (Ceppellini *et al.*, in press). In summary, among a first group of eleven recipients for whom the experiment has been completed, 3, or 27 percent, have developed agglutinins which seemed to be suitable for leukocyte typing. It is proposed to describe in detail the findings on one of these sera, serum Bu, particularly the changes in antibody titer and specificity during the course of immunization. Some observations will also be presented which have a bearing on the understanding of leukoagglutination.

2. Materials and Methods

a. *Leukocyte suspension.* Ten milliliters of blood is withdrawn in a syringe containing 0.5 ml of 10-percent Na_2 EDTA (pH is brought to 6.7 by the addition of NaOH), transferred to a 15-ml tube (125 x 15 mm) and, if necessary, stored at 2°C for up to 24 hr. Both syringe and tube are siliconized (Dow Corning, fluid 200/350). Three milliliters of sterile 3-percent gelatin (Plasmagel, 3) are added (after warming the blood to 37°C). The tube is mixed by two to three inversions (avoiding foaming) and kept at 37°C in a position of 45 deg. for 15 to 20 min., or until a fair sedimentation of the red cells is obtained. The supernatant (usually 5 ml) is withdrawn with a small amount of the upper layer of sedimented erythrocytes, transferred to another siliconized tube (80 x 10 mm) and centrifuged in

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a swingout head on a machine with a finely graduated rheostat. The speed is very slowly increased up to 150 g over 5 min, kept at 150 g for 5 min, and then slowly decreased over 5 min. The supernatant contains the majority of platelets; the sediment, which contains firmly packed red cells under a layer of white cells, is gently resuspended in 0.3 ml of AB human EDTA-plasma diluted to 4:5 with one volume of Plasmagel. It is then transferred to a thin siliconized tube (50 x 6 mm) and kept in a vertical position for 15 to 20 min, or until the red-cell aggregates are sedimented. The supernatant contains the white cells, which are counted and brought to a concentration of 10^7/ml with AB plasma diluted as before. The AB plasma (clarified by centrifugation if necessary) has been selected among patients (from a rheumatoid arthritis clinic) because it offers a very high erythrocyte sedimentation rate and smooth, clean leukocyte suspensions in the absence of specific antibodies. With the use of selected AB plasma, a source of variability, namely the cell donor plasma, is avoided.

Final contamination is 30 to 60 red cells and 300 to 600 platelets per 100 white cells.

b. *Agglutination reaction.* For each test at least five dilutions of antibody (twofold in 0.156 M NaCl) are used. In nonsiliconized tubes a drop (0.03 ml) of leukocyte suspension is mixed with a drop of serum dilution. A third drop is then added, if not differently stated, of 0.156 M NaCl diluted to 4:5 with the following solution: 0.04086 M Na_2 EDTA and 0.04737 M Na_3 EDTA (isotonic, pH 7.38). Incubation is for 90 min at 37°C. The mixture is then transferred to a slide with a siliconized pipette and read at a magnification of 150 x. It is not necessary to lyse the few contaminating red cells with acetic acid.

The results of agglutination are often expressed as a score corresponding to the sum of points arbitrarily assigned to different degrees of agglutination at each serum dilution ++++ 8, +++ 6, ++ 4, + 2, ± 0). When a prozone is present, the same points are given to the first tubes as have been attributed to the strongest reaction following the prozone (e.g., −, +, +++, +++, ++, − = 6, 6, 6, 6, 2, 0 = 26).

c. *Absorptions.* Leukocytes are prepared by sedimentation with Plasmagel, in accordance with the first stage of the method described above. No further purification from red cells and platelets is needed. After counting, the white cells are centrifuged at 1000 g for 15 min, the supernatant discarded, and the cells resuspended in EDTA-buffered saline to a concentration of 10^9 cells/ml. Equal amounts of this suspension and of the serum to be absorbed (undiluted) are mixed and incubated at 37°C for 3 hr with occasional shaking. The mixture is then centrifuged at 1000 g for 15 min; the supernate (absorbed serum 1:2) is then centrifuged at 16,000 g for 30 min before use.

d. *The recipient.* Mr. Bu. is 35 years old and has been secluded for the last 7 years in a psychiatric hospital with the diagnosis of schizophrenia. During a recurrent exacerbation of the mental illness, an intense sitophobia has developed, which requires artificial feeding by canulation. As a consequence, the patient has become anemic (Hb: 7.5 g/100 ml; R.B.C.:3,000,000) and hypoproteinemic (total plasma proteins: 5.2 g/100 ml). His blood group is B, CcDe.

e. *Transfusion schedule.* A donor of group O, CcDe has been selected, solely on the basis of ABO and Rh compatibility. In the course of 18 months, he has given 12 transfusions to the recipient spaced as depicted in Fig. 1. Only the last transfusion (the 13th) has been given from a different donor, for the reasons discussed later. The blood is collected in Fenwal ACD plastic bags and transfused within 12 hr.

No untoward reactions were observed, even when 250 ml of blood with "incompatible" leukocytes were given while the recipient already had developed the leukoagglutinins. The rate of transfusion was always very slow. The treatment was followed by a remarkable improvement of the general and mental condition of the patient.

f. *Serum samples.* Small serum samples were obtained from the recipient at various times during the treatment, distributed in 0.5-ml amounts, and stored at −20°C until used. The samples which have been more extensively investigated have been given a conventional number, prefixed by the letter B, as indicated in Figs. 1 and 2 and Table 1. When the patient's condition was sufficiently good and the characteristics of the serum were of interest, 500 ml of citrated plasma were obtained by plasmapheresis.

g. *Leukocyte panel.* Our panel is composed at present of 120 individuals of group O (both sexes). Each member of the panel gives 20 ml of blood every 4 to 5 weeks; eight individuals are bled each day, 3 days

TABLE 1

Percent of Positive Reactions of Different Samples of Serum Bu, against Leukocytes of the Panel

Serum sample (1)	*Day of bleeding after transfusion*	*Leukocytes tested*	*Percent positive*	*Titer (2)*	*Score (3)*
B/1	60 after 10th	324	56.1	1:8	22
B/2	14 " 11th	203	85.7	1.32	36
B/3	82 " 11th	41	63.4	1:4	20
B/4	15 " 12th	51	90.2	1:16	47
B/5	165 " 12th	93	58.0	1:4	16
B/6	12 " 13th	140	57.1	1:16	32
B/8	36 " 13th	46	58.6	1:64	47

(1) For identification of serum samples see Figs. 1 and 2 and Table 2.
(2) Titer against the leukocyte of the first (specific) donor as reported in Fig. 1.
(3) Mean agglutination score against the six "positive" leukocytes (#1 through #6) of Table 2.

per week. The bleeding is separated into two aliquots, one used immediately, and the second stored as EDTA-blood at 2°C and used within 36 hr for leukocyte separation. Stored blood gives a good leukocyte suspension in 90 percent of cases. By this is meant a reproducibility of the agglutination test not inferior to the one observed between fresh samples bled on different days (Ceppellini *et al.*, in press).

3. Results

a. *Changes in titer.* Figure 1 depicts the changes in titer of the leukocyte-agglutinin(s), tested against the leukocytes of the specific donor. An agglutinating activity had first been observed 7 days after the fifth transfusion (corresponding to a total of 385 ml of whole blood).

The antibody tends to decline rather quickly but it is immediately boosted by a new stimulus. However, a maximum titer of 1:64 has not been surpassed (note the lack of effect on the 10th transfusion). It should be noted that the leukocytes of the specific donor are altogether poor reactors and many positive cells from the general population give higher agglutination scores

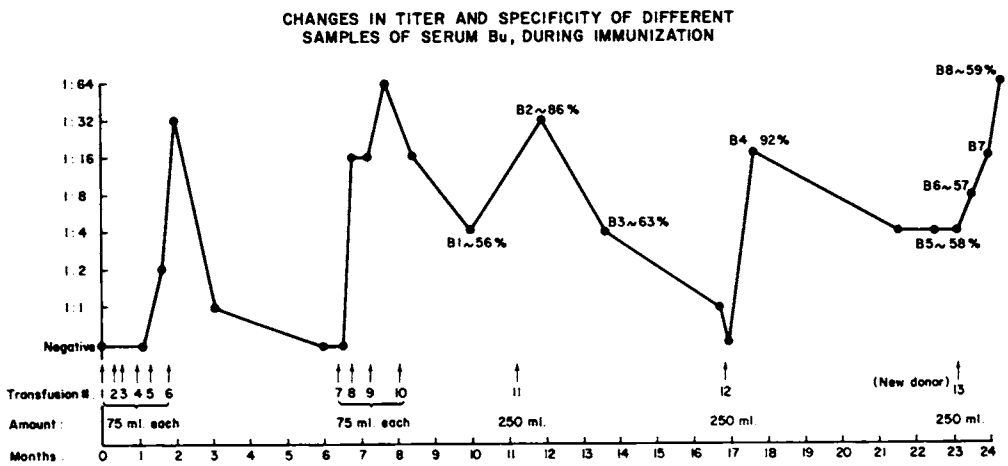

Figure 1.—Titer has been measured against the leukocytes of the first "specific" donor. Specificity is expressed as percent of leukocytes of the panel which are agglutinated. Different serum samples were as indicated in Fig. 2 and in Tables 1 and 2.

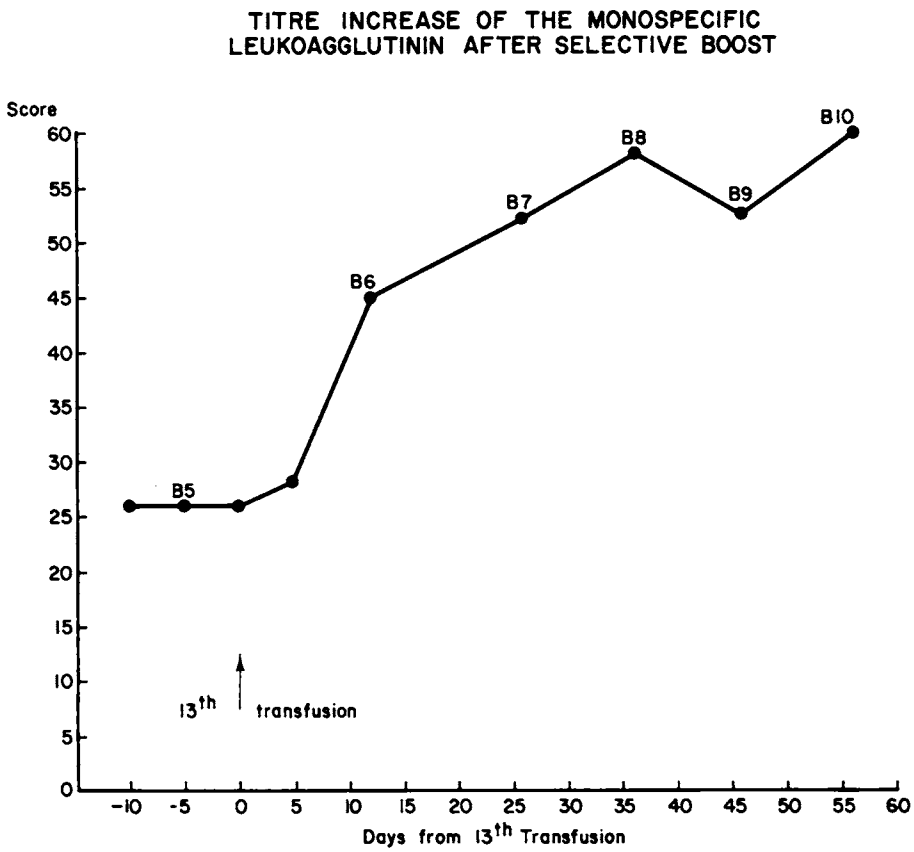

Figure 2.—Thirteenth transfusion (250 ml) from a new donor, positive only for the main antigen (++ 2 of Table 2). Titers are expressed as the average of the agglutination score obtained by titration against four leukocyte samples selected for being B1-positive. Different serum samples were as indicated in Fig. 1 and Tables 1 and 2.

(Table 2). Some samples taken considerably later than the transfusion (e.g., 6 months after the 11th transfusion), did not agglutinate the leukocytes of the specific donor while agglutinating the cells of some individuals of the panel.

b. *Changes in specificity.* The pattern of reactivity of a serum against the cells of a given population is the most important parameter for its characterization. From Tables 1 and 2 it appears that, among the different serum samples which have been sufficiently studied, only B/2 and B/4 differ clearly in specificity from the others. Minor differences among the other samples are discussed in Sec. 3.e.

In an attempt to boost the titer, an 11th transfusion from the specific donor was given, but it instead brought about a dramatic change in the characteristics of the serum. In fact, while sample B/1 agglutinates 56.1 percent of the leukocytes tested, sample B/2 agglutinates 86.2 percent of the cells taken from the same population. All leukocytes which are B/1-positives are also B/2-positives, usually at higher titer than "B/1−; B/2+" cells (see Table 2). Evidently the leukocytes of the specific donor on protracted stimulation have induced the appearance of additional agglutinins directed against different antigenic structures. As shown by absorption and elution experiments (see below), in the case of B/2 and B/4 the alternative explanation can be ruled out, namely, that the same antibody, at a higher titer than in B/1, detects weakly reacting variants of the same antigen.

TABLE 2

Reactivity of Different Samples of Serum Bu Against 14 Selected Leukocytes

*Serum sample**	*Agglutination score against leukocyte number:*													
	#1	*#2*	*#3*	*#4*	*#5*	*#6*	*#7*	*#8*	*#9*	*#10*	*#11*	*#12*	*#13*	*#14*
B/1	16	26	26	22	20	16	8	0	0	0	0	0	0	0
B/2	42	50	64	58	42	38	28	36	42	18	28	16	0	0
B/3	18	28	22	22	16	16	0	6	0	0	10	0	0	0
B/4	36	48	58	56	48	40	36	36	28	26	22	18	8	0
B/5	10	18	22	16	12	16	0	6	0	0	0	0	0	0
B/6	20	38	36	32	28	32	6	4	0	0	0	0	0	0
B/8	38	58	58	42	46	44	22	28	14	16	0	0	0	0
B/10	38	58	50	48	42	44	28	20	20	14	0	0	0	0

* For identification of serum samples see Figs. 1 and 2 and Table 1. Reactivity is expressed as agglutination score on titration. Leukocytes have been selected for being unequivocally "positive" (#1 through #6) or "negative" (#11 through #14) with serum sample B/1, i.e., they gave concordant results in at least five independent tests. Leukocytes #7 through #10 were classified as "doubtful" because they gave discordant results with sample B/1 on repetition of the test; all four, however, were "absorption positive." Leukocytes #1 correspond to the first "specific" donor. Leukocytes #2 correspond to the second donor used for the 13th transfusion.

However, during the usual decline of antibody titer on rest, these additional agglutinins are the first to disappear. In fact, sample B/3 has resumed a specificity similar to that of sample B/1, as shown in Tables 1 and 2.

A new transfusion from the specific donor (the 12th) has induced a similar evolution of the serum. The sample on the 15th post-transfusion day (B/4) gives a slightly higher percentage of positives but its specificity is strongly correlated with the one of B/2 (Tables 1 and 2). The sample at day 165 (B/5), after the 12th transfusion, agglutinates only 58 percent of the leukocytes of the panel.

c. *Stimulation with a different, selected donor.* With the aim of obtaining a high-titer serum which would maintain B/1 specificity, it was decided to transfuse the recipient with blood from a new donor who would share with the specific donor only the major antigenic specificity, while lacking the minor antigens.

For locating such a donor an eluate (E1) was prepared from sample B/4 absorbed into B/1−; B/4+ leukocytes: 4 ml of undiluted B/4 were incubated with 4 x 10^9 leukocytes from four different B/1−; B/4+ individuals. After 4 hr at 37°C the supernatant was discarded (it showed B/1 specificity) and the cells washed four times with cold phosphate-buffered saline. They were then resuspended in 1 ml of AB serum diluted 1:2 with EDTA–buffered saline and incubated at 56°C for 10 min. The mixture was then centrifuged at 1000 g in warm cups (56°C), the supernatant was removed and the mixture centrifuged at 30,000 g. This eluate agglutinates about 60 percent of the cells of the population, independently of their B/1 reactivity.

With this reagent a donor was selected as being B/1+; E1−. A transfusion of 250 ml of whole blood boosted the titer of the serum against the leukocyte of the first (specific) donor from 1:4 to 1:64 in the course of 3 weeks (Figs. 1 and 2), but this time, at variance with the effect of transfusions 11 and 12, specificity remained near the 56-percent level (Table 1). Two months after transfusion the titer had not yet shown a decrease.

d. *Monospecificity of the B/56%-58% samples.* The presence of more than one antibody in samples B/2 and B/4 has been clearly demonstrated also by absorption experiments. As shown in Table 4, sample B/4 contains at least two additional antibodies, besides the one present in B/1. On the contrary, samples B/1, B/8, and, by inference, the other samples with specificity around 58 percent, seem to contain only one antibody directed against a single antigenic factor (Table 3).

The data reported in Table 2 at first glance suggest that B/8 and B/10 differ from B/1 and B/6 because leukocytes #9 and #10 are agglutinated only by the former but not by the latter samples. It should, however, be noted that leukocytes #9 and #10 are nonetheless able to absorb out completely the antibody present in B/1 and B/6. Thus, in the tests represented in Tables 2 and 3, they behave with regard to these two sera as "agglutination-negative-absorption-positive" (ANAP) cells, already described by van Rood (1962).

e. *Reproducibility of leukoagglutination.* Leukocytes #9 and #10, as well as #7 and #8, in five different repetitions of the test on cells obtained by different bleedings, gave discordant results against sample B/1, being sometimes negative and sometimes positive (at low titer). In fact, they were selected as being "doubtful" for the experiments presented in Tables 2 and 3. On the contrary, leukocytes #1 through #6 (the "positives") were agglutinated by sample B/1 five times out of five. Leukocytes #11 through #14 (the "negatives") gave a negative reaction five times out of five and were not able to absorb out the B/1 antibody even when used in excess (10^9 cells/ml of serum).

Thus, it is suggested that nonreproducibility and ANAP are related phenomena and may depend on some peculiarities of the antigen (weak variants like the Rh D^u), and on the characteristics of the antiserum. It is possible that the ability of B/8 and B/10 samples to agglutinate cells which are not consistently agglutinated by B/1 and B/6 are attributable to their higher titer. In any case, this represents one other source of variability which contributes to the almost infinite diversity of leukoagglutinating sera.

Reproducibility of sample B/1 is 95 percent (Ceppellini *et al.*, in press). Reproducibility of samples B/8 and B/10 has not yet been systematically explored, but on the basis of what has been said, it should be still higher.

f. *Sensitivity of the leukoantigen BU to pH and temperature.* A number of authors (Amos and Peacocke, in press; Killmann, 1959) have reported that an excess of EDTA (above 0.15-percent final concentration) inhibits leukoagglutination even in the variant of the test, such as the one here used, where enough chelating agent is present to exclude the participation of complement (the LAS test of Dausset, 1962). While investigating this phenomenon, however, we recognized that the addition of one volume of Na_2 EDTA to the reacting mixture lowers the pH, and at salt concentrations above 1 percent the buffers present in the system become inefficient. Thus the addition of a drop of 5-percent Na_2 EDTA in water (isotonic) to a drop of cell suspension and a drop of serum dilution brings the pH below 5 and

TABLE 3

Cross-Absorption Experiments with Sample B/1

		Reaction of leukocyte number:									
		#1	*#2*	*#3*	*#4*	*#7*	*#8*	*#9*	*#10*	*#11*	*#14*
Unabsorbed serum		+++	+++	+++	+++	±	±	−	−	−	−
Serum absorbed with leukocytes	#1	−	−	−	−	−	−	−	−	−	−
	#2	−	−	−	−	−	−	−	−	−	−
	#3	−	−	−	−	−	−	−	−	−	−
	#4	−	−	−	−	−	−	−	−	−	−
	#7	−	−	−	−	−	−	−	−	−	−
	#8	−	−	−	−	−	−	−	−	−	−
	#9	−	−	−	−	−	−	−	−	−	−
	#10	−	−	−	−	−	−	−	−	−	−
	#11	+++	+++	+++	+++	++	+	−	±	−	−
	#14	+++	+++	+++	+++	+	−	−	−	−	−

For absorption technique see text. Leukocyte donors correspond to the ones used for the experiments presented in Tables 2 and 4. Note that after absorption with "negative" cells, the reactivity of serum against "doubtful" is sometimes increased. Cross-absorption of sample B/8 gave the same pattern.

TABLE 4

Cross-Absorption Experiments with Sample B/4

		Reaction of leukocyte number:									
		#1 abc	*#2 a*	*#3 ab*	*#4 ac*	*#7 abc*	*#8 ab*	*#9 a*	*#10 ac*	*#11 bc*	*#14*
Unabsorbed serum		+++	+++	+++	+++	+++	+++	++	++	++	−
Serum absorbed with leukocytes	#1	−	−	−	−	−	−	−	−	−	−
	#2	++	−	++	+	++	+	−	±	++	−
	#3	±	−	−	+	±	−	−	±	+	−
	#4	++	−	++	−	+	++	−	−	+	−
	#7	−	−	−	−	−	−	−	−	−	−
	#8	+	−	−	+	+	−	−	+	+	−
	#9	++	−	+	+	++	+	−	+	++	−
	#10	+	−	++	−	++	+	−	−	++	−
	#11	+++	+++	+++	++	++	++	++	+	−	−
	#14	+++	+++	+++	++	++	+++	++	++	++	−

For absorption technique see text. Leukocyte donors correspond to the ones used for the experiments presented in Tables 2 and 3; abc represent hypothetical discrete antigens attributed to the different leukocytes for explaining the results, on the assumption that sample B/4 contains at least three different leukoagglutinins. #1 is the first (specific) donor; #2 is the donor selected for the 13th transfusion.

completely inhibits the reaction. If, however, the EDTA solution is neutralized with Na-OH, then even at a 5-percent concentration, it does not interfere with leukoagglutination. The same inhibitory effect is also obtained by addition of acetic acid/Na acetate buffer at a pH of 4.5.

To determine whether pH interferes with the antigen-antibody fixation or with the cell surface, leukocyte suspensions were incubated for 1 hr at 37°C with one volume of different Na_2 EDTA and acetic acid/Na acetate isotonic solutions. These mixtures were then centrifuged for 5 min at 150 g, the supernatant was removed and substituted with AB plasma gelatin, and the agglutination test was carried on as usual. The results of this experiment are presented in Table 5.

To determine whether acid-treated leukocytes, no longer agglutinable, were nonetheless able to fix the antibody, 10^6 cells/ml were treated for 1 hr at 37°C with different reagents, then washed and used for absorbing 1 ml of antiserum, which was then titrated against normal positive cells. As shown in Table 7, acid-treated cells are unable to absorb out the antibody.

Preheating of the cells at 45°C or above also has an inhibitory effect on leukoagglutination (Table 6). Leukocytes heated at 45°C for 20 min, however, are still able to absorb the antibody, although less efficiently than untreated cells.

4. Genetics of Antigen BU

If it is accepted that samples of serum Bu with a specificity of 56 to 58 percent are monospecific, they detect a corresponding antigen BU and can be used for genetic studies. Preliminary family data are in agreement with the hypothesis that antigen BU is inherited, as are the great majority of blood antigens, as a monofactorial haplosufficient trait. In fact, from five BU-positive/BU-negative matings, out of 12 children observed, six belonged to the one and six to the other parental phenotype. On the basis of the frequency of the BU-positives in the general population, the gene frequencies are 0.35 for the "positive" allele and 0.65 for the "silent" allele (or alleles). Among the 58 percent BU-positives, about 45.6 percent are expected to be heterozygous and 12.4 percent homozygous. The cells of three members of one family were "doubtful" BU-positive; they were not agglutinated (or only weakly and inconsistently) by samples B/1 and B/6, but could absorb out the antibody. They were however consistently agglutinated by samples B/8 and B/10. These leukocytes may correspond to a weak inherited variant of antigen BU.

Antigen BU is independent of system 4 of van Rood and van Leeuwen (1963). It has not been possible to compare it with antigen Mac, which has a similar frequency in the French population and it seems to elicit not rarely the corresponding antibody in polytransfused patients (Marchal *et al.*, 1958). Agglutinins detecting BU have been found, mixed with additional antibodies, in two other patients.

5. Discussion

In a recipient of multiple transfusions, the chances of developing antibodies directed against different white-cell antigens are high,

TABLE 5
Effect of EDTA and pH on Leukocyte Agglutinability

Pretreatment of leukocytes (1 hr at 37°C)		*Agglutination score*
Buffer and concentration	*pH*	
Na_2 EDTA 0.2%	6.8	34
Na_2 EDTA 1%	6.8	36
Na_2 EDTA 3%	6.8	38
Na_2 EDTA 5%	6.8	30
Na_2 EDTA 5%	4.5	0
acetic ac./Na acetate	4.5	0
acetic ac./Na acetate	5.5	18

For explanation on pretreatment see text. Agglutination is expressed as a score which is the average of the reaction strength obtained with four different positive leukocytes. Serum sample B/6 was used.

TABLE 6
Effect of Temperature on Leukocyte Agglutinability

Temperature	*Time, min*		
	5	*15*	*30*
37°C			32
41°C	29	27	31
45°C	20	0	0
56°C	0	0	(*)

Leukocyte suspensions in buffered saline were warmed at the temperature and length of time indicated, they then were brought to 37°C, the antibody dilutions added, and the reaction carried on as usual. Strength of the reaction is expressed as the average score obtained with three different positive leukocytes. Serum B/6 was used. After 30 min of incubation at 56°C (*) the suspensions showed cell lysis and nonspecific clumping in the controls.

TABLE 7
Effect of Pretreatment on the Capacity of Leukocytes to Fix Antibody

Pretreatment of leukocytes used for absorption				*Agglutination score*
Unabsorbed serum				38
After absorption with leukocytes (10^6/ml) pretreated:				
Time	*Temp.*	*Buffer*	*pH*	
1 hr	37°C	saline	7.2	0
20 min	45°C	saline	7.2	0
20 min	56°C	saline	7.2	26
1 hr	37°C	Na_2 EDTA 5%	6.8	0
1 hr	37°C	Na_2 EDTA 5%	4.5	18
1 hr	37°C	Ca EDTA 5% (*)	4.0	20
1 hr	37°C	Na acet./acet. ac.	4.5	24
1 hr	37°C	Na acet./acet. ac.	5.5	10

For explanation of pretreatment see text. After absorption the serum (B/6) was titrated against four different positive leukocytes. The agglutination results are expressed as the average score. (*) Chelating power of Na_2 EDTA exhausted by addition of $CaCl_2$.

even if the blood has been obtained from a single donor. The possible reasons for the frequent coexistence of multiple antibodies in the same serum have been discussed elsewhere (Ceppellini *et al.*, in press). However, the different antibodies have evolutions independent of each other. Some, in fact, appear sooner, reach a higher titer, and remain longer, even in the absence of further stimulation. Thus the chances of finding monospecific sera are greater at the beginning of immunization or after a long period of rest.

What is more important from the practical point of view is that the different antibodies respond specifically to secondary stimuli. Thus after repeated transfusions from a single donor, which resulted in the production of multiple antibodies, it has been possible to raise specifically the titer of the antibody against a given antigen, by selecting a new donor who differs from the recipient in only one of the antigens detected by the various antibodies. Nonspecific anamnestic response was not observed.

The lack of reproducibility of leukocyte agglutination is a major practical difficulty

since an individual's leukocytes, once typed as positive (or negative), may become negative (or positive) on a second trial with the same serum (Ceppellini *et al.*, in press). In the majority of cases tested (where nonspecific clumping was absent from control suspensions), leukocytes which gave contrasting results on repetition of the test were shown to be able to absorb the antibody. Although such findings suggest that in general the negative reaction is the false one, it also suggests that nonreproducibility is related to the phenomenon described by van Rood (1962) as the ability of some leukocytes to fix the antibody while not being agglutinated.

This characteristic is peculiar to leukocytes of certain individuals and is possibly inherited, but it also depends on some properties of the serum used. In fact, samples of serum having the same single specificity showed different efficiency in agglutinating these particular cells. The titer seemed to be an important factor, but some qualitative characteristics of sera obtained after hyperimmunization, perhaps representing a better fit between the antibody and the determinant groupings of the antigen, may play a role. By inference, high-titered sera should give a better reproducibility of leukoagglutination.

During this study, it was also found that high concentrations of EDTA (above 1.5 percent in the final mixture) do not adversely affect the test, if pH has been kept around 7. Not only Na_2 EDTA but also Na acetate, when the pH is below 4.5, irreversibly alters the leukocyte antigen, which is then no longer able to fix the antibody. Thus this effect seems to be due to pH and not to sequestration of bivalent ions. Warming the cells for 15 min at 45°C also makes them unagglutinable. These observations, if systematically extended, may lead to a better understanding of the physicochemical characteristics of the antigen and of the cell surface.

The antigen detected by the monospecific samples of serum Bu has been given the provisional name BU. In the population of Turin it has a frequency of 58 percent. Family studies have shown that it is inherited as a monofactorial, haplosufficient trait. It has been shown not to correspond to the two antigens a and b of system 4 of van Rood and van Leeuwen (1963). Should it be found not to belong to any of the other systems which have been recently identified (Payne and Hackel, 1961; van Rood and van Leeuwen, this conference), an attempt will be made to produce antibodies detecting its allelic antigen or antigens. On the basis of calculated gene frequencies, one out of five BU-positive individuals are expected to be homozygous, and hence potentially able to produce antibodies against the corresponding allelic antigen(s) when transfused with BU-negative blood.

In fact, the identification of the various allelic antigens is of great practical, as well as theoretical, importance. As emphasized by van Rood and co-workers (in press), whose brilliant work has opened a new lead into the problem, only a complete analysis of each individual genetic system of leukoantigens will provide a reliable and fool-proof evaluation of the relevance they may have for homotransplantation, and possibly lead to the identification of some of them with a major histocompatibility system.

The data here presented suggest that "planned" immunization of recipients with selected bloods offer a practical means of coping with the problem of obtaining strong specific antibodies for use in tissue typing.

6. Summary

Leukoagglutinins present in different serum samples of a recipient who received multiple transfusions from selected donors have been carefully investigated during the course of the immunization process. The dynamics of the antibody response and the changes in titer and specificity have been studied. Different antibodies coexisting in the serum respond specifically to secondary stimuli. Thus it has been possible to obtain a high-titer monospecific serum which detects a leukocyte antigen (BU) present in 58 percent of the population. This antigen is easily inactivated by low pH and heating. The significance of leukoagglutinins for tissue typing has been discussed.

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Discussion

DR. KREVANS: It has been suggested that you cannot make a direct translation between humoral antibody and tissue rejection. In line with Dr. Ceppellini's observation, I would like to report very briefly on some observations on red-cell antigens. We attempted to immunize a group of Rh-negative people with Rh-positive cells and by the type of experiment just discussed we searched for antibody as a sign of successful immunization. With the particular dose of red cells we were using and the particular time interval of injection, we found what others have found many times. With one injection you can immunize about 10 percent; after two, some 22 percent; and on up, after five injections about 50 percent of our original group was immunized. You would interpret this as saying the first group was immunized with one, the second group after two injections, etc., as was suggested here. But during the course of these experiments, we were doing the immunization with chromium-labeled cells, so that we had survival data following each of these injections. What we found in fact was that, with only one exception, all of our 50 percent who eventually were immunized showed immune clearance from the second injection on. So that we would have a series of curves in which the first injection gave us normal survival for the first 7 days, the second injection would give us a survival of this sort (rapid clearance), the third injection and the fourth injection gave similar curves, and only after the fourth injection was antibody detected. So that at least for the red-cell "rejection system" we had, I think, very impressive evidence that the failure to demonstrate humoral antibody did not indicate that immunization, as measured by tissue rejection, had not taken place.

DR. AMOS: What about those that did not subsequently make antibody?

DR. KREVANS: As I say, we now have only one recipient who made antibody after the third injection but did not have immune clearance following the second injection. The suggestion is that you are either immunizable to the Rh antigen or you are not with this particular dose of red blood cells.

DR. DAUSSET: I want to make a comment regarding differences observed when using different techniques of leukoagglutination. When one uses a defibrinated-blood instead

of an EDTA-blood technique one does not get always the same specificity. We studied also the Buffo serum provided by Dr. Ceppellini. It gave a greater number of positive reactions with the defibrinated-blood technique in which the complement or a complement-like factor is present, than with the EDTA-blood technique.

DR. CEPPELLINI: On the other hand, Dr. Shulman has tested some Buffo serum and was not able to get complement fixation.

DR. SHULMAN: That is right.

DR. CEPPELLINI: And I may add that not all the sera are 19S. Most of them were 7S, but probably a few had the kind of high molecular weight of the isoagglutinins described by Dr. Kunkel.

DR. SHULMAN: I think we should mention that the anti-4A and anti-4B sera did not fix complement either.

Sero-Typing of Human Lymphocyte Antigens: Preliminary Trials on Long-Term Kidney Homograft Survivors*

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The rationale behind attempts to select proper donors for homotransplantation has been aptly given recently by many authors (Hamburger *et al.*, 1962; Brent and Medawar, 1963; Rogers, 1963; Wilson, Henry, and Merrill, 1963; Marchioro *et al.*, 1964; Starzl *et al.*, 1964a). How this might be accomplished is the immediate concern of this conference. In this preliminary study we have investigated the possibility of using allogenic antisera derived from women with multiple abortions or pregnancies as reagents to detect transplantation antigens presumably possessed by indicator blood lymphocytes. That the resulting serologic reaction would reveal the major transplantation antigens is the prime premise of this approach. Although experiments with mice have provided evidence that strong transplantation antigens are demonstrable serologically (Gorer, 1956; Brent, Medawar, and Ruszkiewicz, 1961), and that the reaction against minor antigens can be suppressed (Shapiro *et al.*, 1961; McKhann, 1962; Medawar, 1963), there is no evidence that an analogous situation exists in man.

As the initial step in testing the efficacy of the lymphocyte cytotoxic reaction as a typing procedure for homotransplantation, long-term kidney homograft survivors and their donors were tested for compatibility. If fortuitous matching of transplantation antigens had been principally responsible for the long survival of renal transplants in these selected patients, a reasonable degree of compatibility should be evident from the typing studies. Precise correlation of survival time with typing methods cannot be expected, since the method of immunosuppression was regulated by the clinical course of individual patients. However, if the matching of antigens, particularly by the device of obtaining grafts from related donors, had played an important role in long-term survival, a distinct trend in matching should be evident. In the present provisional trials, such a trend was obtained.

Methods

The methods of transplantation in adults and children (Starzl *et al.*, in press, a, b), as well as techniques of nephrectomy (Marchioro *et al.*, 1964) and perfusion (Starzl *et al.*, 1963), have been reported earlier. A synopsis of the case histories of 12 patients from the Denver Veterans Hospital and the University of Colorado School of Medicine is given in Table 1. Summaries of two long-term survivors of kidney transplants at the UCLA Medical Center (Goldman *et al.*, submitted for publication) are shown in Table 2.

Heparinized blood (20 to 50 units/ml) obtained from kidney donors and recipients in Denver was flown to Los Angeles. Viable lymphocytes were isolated within 12 hr from the time of bleeding. The method of isolation consisted of differential adherence of granulocytes on a polystyrene surface followed by centrifugation in capillary tubes to eliminate most of the erythrocytes (Terasaki *et al.*, in press; Terasaki and McClelland, submitted for publication). Lymphocyte antigens were identified by cytotoxicity of 50 different human antisera. The sera were obtained mainly from women with multiple abortions and pregnancies; a few were obtained from multiple transfusions (#2, 6, and 15), and two from persons immunized by

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TABLE 1

Summarized Case Histories of 12 Renal Transplant Patients from the Univ. of Colorado School of Medicine and Denver V.A. Hospital

Recip.	*Donor Relation*	*Time since Transpl.*	*+ or − days of Transpl.*		*Ischemia Time, min*	*Reject., Days P.O.*	*Therapy for Reversal**	*Current*	
			Splenect.	*Nephrect.*				*BUN*	*Creat. Clear.*
**1	Mother	18 mo.	−20	−20	34	12	Azathiop., Pred., Act. C	15	91.3
2	Brother	13 mo.	−26	0, +7	35	18	Pred., Act. C	16	114
4	Brother	10 mo.	0	0	22	1½	Pred., Act. C	33	86.8
5	Mother	9 mo.	−21	−21	37	21	Pred., Act. C	28	76
6	Mother	7 mo.	0	0	42	5	Pred., Act. C	22	51.6
7	Wife	7 mo.	0	0	38	1	Pred., Act. C	16	101
9	Mother	6 mo.	0	0	18	10	Pred., Act. C	21	77.6
10	Mother	6 mo.	0	0	27	12	Pred., Act. C	19	61.1
11	Father	6 mo.	0	0	26	4	Pred., Act. C	21	101.5
***12	Aunt	3 mo.	0	0	25	30	Local X-ray, Act. C	40	96.5
***13	Father	3 mo.	0	0	27	3	Local X-ray, Act. C	32	112
***14	Mother	3 mo.	0	0	30	2	Local X-ray, Act. C	19	114

* Azathiop. = azathioprine; Pred. = prednisone; Act. = actinomycin.

Patients 1 and 2 were thymectomized 1 mo. before transplantation.

All patients except 1 and 13 were treated with azathioprine for 10 days preoperatively in doses of 1 to 3 (mg/kg)/day. Postoperatively, azathioprine was continued in doses of 2 to 6 (mg/kg)/day, the exact dose being determined by the peripheral white cell count on the day of drug administration. Patient 13 was preforcated for 1 day only.

** Immunosuppression consisted of 300 r total body radiation preoperatively and 100 r total body radiation postoperatively, administered in divided doses. With the onset of rejection, azathioprine was added to the regimen and continued in doses of 2.5 to 4 (mg/kg)/day.

*** These three patients received 150 to 200 mg of prednisone per day preoperatively for 1 to 2 days in addition to pretreatment with azathioprine. Both drugs were continued postoperatively. Prednisone dosage has been systematically decreased in each case in accordance with renal function. Local irradiation, 150 r to the transplant, was used to reverse rejection crises. It was delivered on 3 alternate days for a total dose of 450 r per course of therapy. The last two patients received two full courses of X-ray. The patient 12 has had only one course. Prednisone is no longer taken by 1 or 2.

TABLE 2

Summarized Case Histories of Two Renal Transplant Patients from UCLA (Drs. Goodwin, Martin, Kaufman, and Goldman)

Recip.	Donor Relation	Time since Transpl.	+ or − days of Transpl.		Ischemia Time, min	Reject., Days P.O.	Therapy for Reversal*	Current	
			Splenect.	Nephrect.				BUN	Creat. Clear.
3	non-related	12 mo.	not done	0	45	10	Azathiop., Pred., Act. D	19	43
8	Mother	6 mo.	0	0	40	40	Azathiop., Pred., Act. C	15	41

* Azathiop. = azathioprine; Pred. = prednisone; Act. = actinomycin

three or four consecutive skin homografts (#22 and 24).* Antisera #15, 27, and 14 were from habitual aborters.**

The microdroplet oil chamber method of testing has been described (Terasaki and McClelland, submitted for publication). Essentially, mixtures of 2,000 lymphocytes, 0.003 ml of absorbed rabbit complement, and antibody dilutions by thirds from 0.003 to 0.00003 ml were incubated for 4 hr at room temperature. The microreactions were accomplished in an oil chamber and assayed by counting the percentage of viable cells under phase-contrast microscopy at 620 × or 820 × magnification.

Erythrocytes were typed with standard commercial antisera, using the Coulter Counter to measure agglutination (Terasaki *et al.*, in press).

Results

Red-cell antigens. Many of the long-term surviving kidney homograft patients have survived despite one to three "minor" erythrocyte-group incompatibilities with their respective donors (Fig. 1). Most erythrocyte groups seemed innocuous, for incompatibilities were noted with E, Fy^a, M, C, D, s, and c. Among the limited numbers of survivors tested, no incompatibilities were found for A, B, N, or e. The fact that eight out of 11 of the 6-months-or-longer group possessed one incompatibility or none may suggest, however, that multiple incompatibilities may be deleterious. On the other hand, since two of the longest survivors had two or three incompatibilities, mismatched erythrocyte antigens may not be a critical factor to survival of the graft. In patient #4, in whom there was a C and D mismatch, 10 months after transplantation antibodies were detected against donor erythrocytes. If it is assumed that no transfusions of Rh^+ blood were given, these antibodies may have been elicited by antigens in the donor kidney. The fact that the kidney is functioning 10 months after transplantation demonstrates that the presence of such antibodies is not clinically deleterious to the kidney graft. Similar tests on papainized erythrocytes of the donor with the plasma of recipients #1, 2, 12, 14, 13, and 10 were negative. In these

* Kindly contributed by Dr. Roy Walford; #22 and 24 are designated Bu and Mo, respectively, in Walford, Gallagher, and Sjaarda (1964).
** Generously contributed by Dr. Phillip Levine.

patients an indirect Coombs test using the donor erythrocytes was also negative.

Lymphocyte typing. Cytotoxicity of antisera dilutions were recorded as approximate percentages of cells killed and plotted for each donor and recipient pair as shown in Fig. 2. This example illustrates the reactions of the donor (mother) and recipient #9. By comparison of the cytotoxicity of each antiserum horizontally between the donor and recipient, it can be seen that antigens 5, 17, 22, 25, 41, 44, 45, 48, and 53 are present in the donor but lacking in the recipient. These antigens may be considered to be foreign to the host, and therefore immunogenic. Although identity tests of these antisera are not yet complete, it would appear that incompatibilities detected by these eight antisera are not completely deleterious, for with the immunosuppressive treatments used (Table 1), the patient is alive and well 6 months postoperatively.

One example in which the donor antigens were quite dissimiliar to the recipient's antigens but were in almost all instances *less* than the recipient's is given in Fig. 3. In this pair, the donor and recipient were *not* related (wife to patient #7). According to the lymphotoxicity reactions, only one incompatibility with antiserum 16 was noted.

Results of titrations as given in Figs. 2 and 3 are summarized in Fig. 4 by plotting only those antigens that were incompatible. A "unit" was taken as the difference in dilution "droplet" at which comparable reactions were obtained. One unit difference therefore corresponds to a three-fold disparity in volume of antisera necessary to kill, since the antisera were diluted by 1:3. The maximum of five units corresponds to a 243-fold antiserum volume difference. Since earlier unpublished work on inbred mice has indicated that quantitative differences obtained with presumably polyvalent unabsorbed antisera may reveal qualitative variances, some attempt was made to indicate the extent of incompatibility if the host possessed measurable antigens. Thus a distinction is made between a complete incompatibility in which the recipient lacks all detectable antigens (indicated by the solid bars) and partial incompatibility in which the recipient possesses some antigenic activity but at a level lower than the donor (indicated by the striped bars). Complete incompatibilities, which would be deemed detrimental to a graft, were found to be slightly more predominant in the recipients who are living 3 to 6 months than in those living 6 to 18 months. Partial incompatibilities were more randomly distributed. Perhaps significantly, no complete incompatibility was found in patient #1, who has lived 18 months after the operation. Of the patients tested, the number of mismatched antigens was the largest in one 6-month survivor (#9) and one 3-month survivor (#12).

As an initial attempt to devise criteria by which degrees of incompatibility can be assessed, the following test was applied. Taking four long-term survivors as recipients, 16 individuals who were kidney donors or recipients were tested as hypothetical donors. Every combination of donor-to-recipient was examined for lymphocyte antigen incompatibility and plotted by four methods (Tables 3 and 4). In Table 3, the actual *number* of antigens which were mismatched was totaled. "Complete" incompatibilities, as defined earlier, and "partial" incompatibilities were added separately and noted; the trial donors with the lowest number of incompatibilities appear toward the left. Significantly, the actual donors used, 1D, 2D, 6D, and 7D, fell within the first two columns, whereas many other possible donors possessed six or more incompatible antigens. Individual #14 can be seen to be the worst possible donor for all the recipients, whereas individual 7D (shown in Fig. 3) would have been a universal donor according to this scheme. If dissimilarities of up to five antigens can be suppressed with pharmacotherapy, the actual donor (1D—mother) or 7D could have been used as a donor for recipient #1 and 15 other persons could have been unsuitable. For recipient #2, out of 16 possible donors, 11 might have been used. Summation of partial incompatibilities yielded somewhat similar results in showing certain individuals to be better donors than others.

If the difference in *units* of mismatch are totaled as in Table 4, a greater spread in the range of incompatibility is obtained. Increased specificity in selection of donor seems to be gained, for the actual donors 1D, 2D, 7D, and 8D can be seen to be good donors for some recipients, but excessive-antigen donors for others. For example donor 2D possessed 13 excess units (u) for #1, 5u for #2 (the actual recipient), 6u for #7, and 9u for #8.

In the course of these studies, an interesting observation was made on one patient (of Drs. Goodwin, Martin, Kaufman, and Goldman) who suffered an "immediate rejection" of a kidney transplant. The kidney, trans-

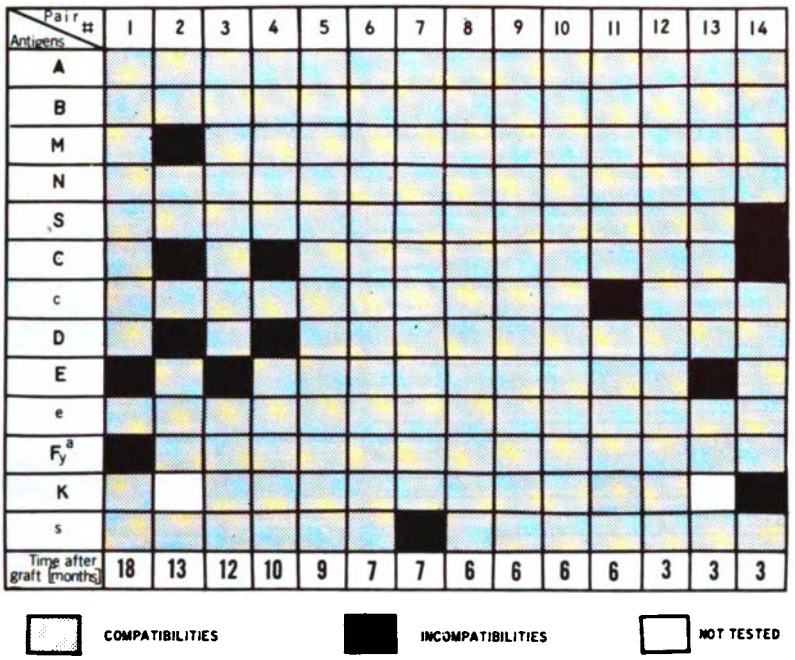

Figure 1.—Erythrocyte-group incompatibilities between kidney homograft donors and their recipients are shown in the dark bars.

planted from a brother to sister, remained anuric for 2 weeks, after which it was removed. Tests of the recipient's serum *before* grafting revealed cytotoxic antibodies against the donor's lymphocytes. Serum taken on the day of transplantation just before grafting was cytotoxic to 70 percent of cells (using 0.003 ml of serum, 0.005 ml of C′, and 2,000 lymphocytes) and became slightly less toxic 2 and 6 days after grafting. These antibodies could have been elicited by transfusions and may be suspected of being responsible for the immediate rejection.

Discussion

The preliminary results described here suggest that lymphocyte cytotoxicity may be used to detect and match transplantation antigens. The fact that relatively few incompatibilities were found between the lymphocyte antigens of the donor and of the kidney-graft recipients who have survived for 6 to 18 months (Fig. 4) may be taken to indicate that this underlying tissue compatibility was an important contributory factor in the successful outcome. Obviously, matching alone is not a *sufficient* condition, for numerous factors, such as low ischemia times and proper diagnosis and therapy of "rejection" crises, are essential (Starzl *et al.*, 1964b). A lesser degree of matching presumably would have led to rapid rejection. The possibility that the apparent correspondence was merely the result of similar but nonspecific reactions of the antisera was tested by comparing the typing reactions of random hypothetical donors with the donor actually used. In most instances, the actual donor was found to be one of the better donors with few incompatibilities, whereas many other donors possessed numerous antigens in excess of the recipient (Table 3). Of considerable significance is the fact that for some recipients a nonrelated individual was an even more suitable donor than the close relative used. Persons who lacked many antigens, such as the wife of recipient #7, may be almost universal donors. Others, such as individual #14, would have been undesirable donors for all the recipients tested.

An apparent degree of matching may also have been produced by the action of numerous antibodies of varying specificities present in a reagent antiserum. Thus two cells possessing different antigens may be killed by the same antiserum (containing several antibodies) and therefore may appear as cells

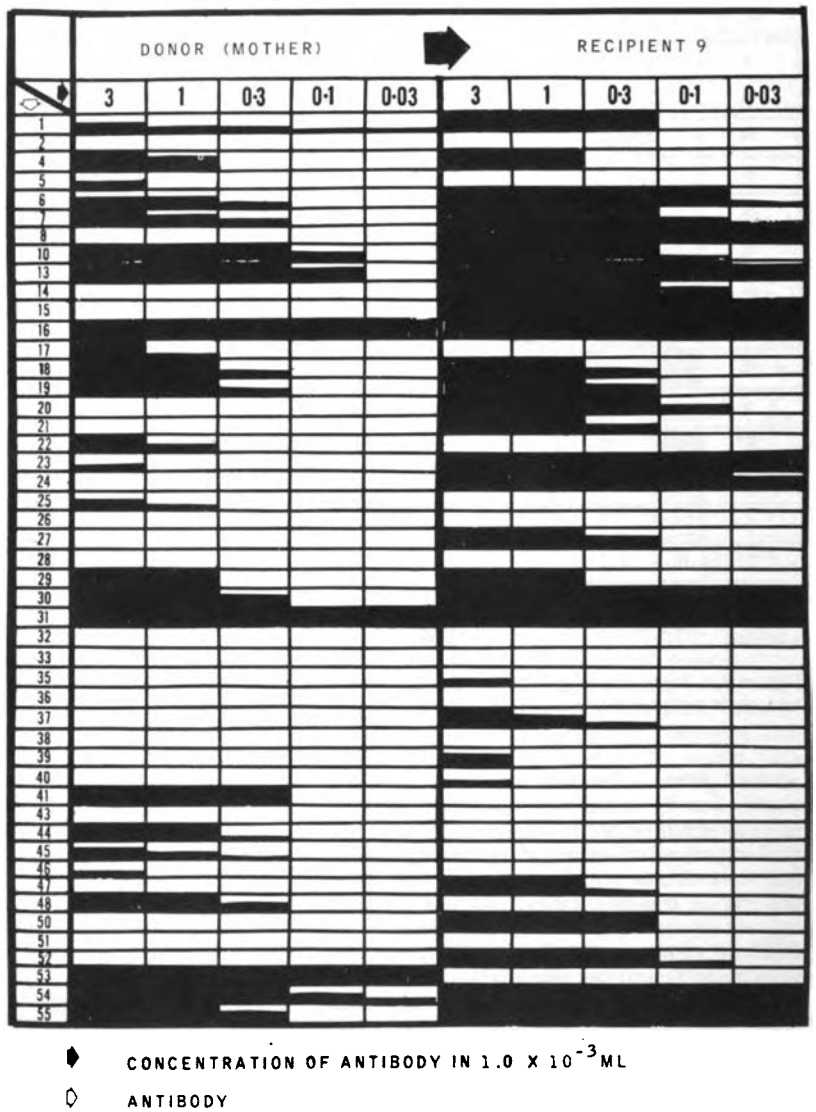

Figure 2.—Lymphocyte-cytotoxicity titration of the donor (mother) and recipient (#9). The antisera are listed on the left and volumes of the antisera tested X 10^{-3} ml are given in the top row of figures. The approximate extent of cytotoxicity at each dilution is denoted by the height of the dark bars. Incompatibilities are present wherever the donor (shown on the left) reacts with a serum and the recipient does not.

having identical antigens. This difficulty can be circumvented, at least theoretically, by absorbing the sera with recipient cells and testing with the donor cells (Becker and Terasaki, submitted for publication). In a few instances in which this procedure was adopted for all reactions for which the donor and recipient were positive, no new specificity could be uncovered. As a temporary expedient until "purified" antisera are produced, it was also anticipated that, by testing with numerous antisera, differences not revealed by one "mixture" may be detected by another.

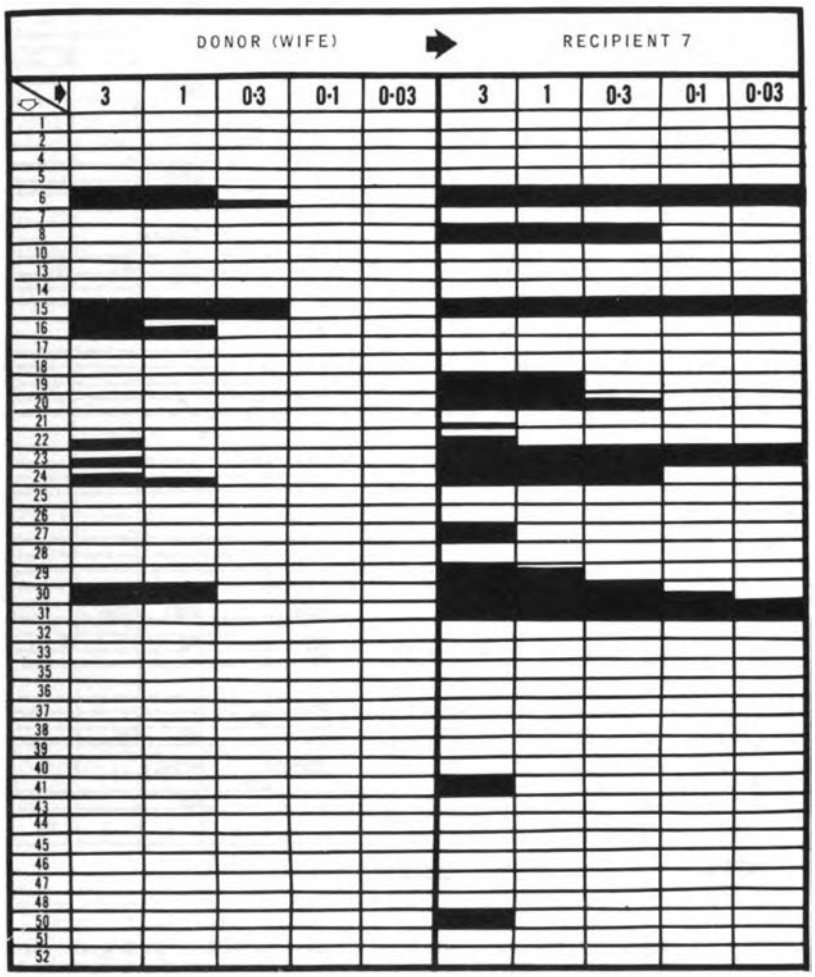

Figure 3.—Lymphocyte-cytotoxicity titration of a donor (wife) and unrelated recipient (#7). The results are plotted as in Fig. 2. Of particular interest is the fact that the donor possessed only one antigen that the recipient did not, whereas a graft in the opposite direction would have been incompatible for 8 antigens.

An incompatibility in which the donor reacts with a given antiserum and the recipient does not is not open to the same polyvalent-antiserum criticism. Cells of the donor may react on the basis of A, B, C, . . . Z antigens, whereas the recipient can be taken to lack all these antigens from a negative reaction. This type of incompatibility, however, does suffer from another difficulty, in that a non-reactive cell may be capable at times of absorbing out cytotoxicity (Becker and Terasaki, submitted for publication; Terasaki *et al.*, in press). Van Rood has earlier encountered a similar phenomenon with leuko-agglutinins which he termed agglutination-negative-absorption-positive reaction van Rood and van Leeuwen, 1963), and similar reactions have been found with erythrocytes (Race and Sanger, 1962). Absorption experiments may therefore be essential to prove that an antigen is lacking, for the amount of antigen present may be sufficient to absorb

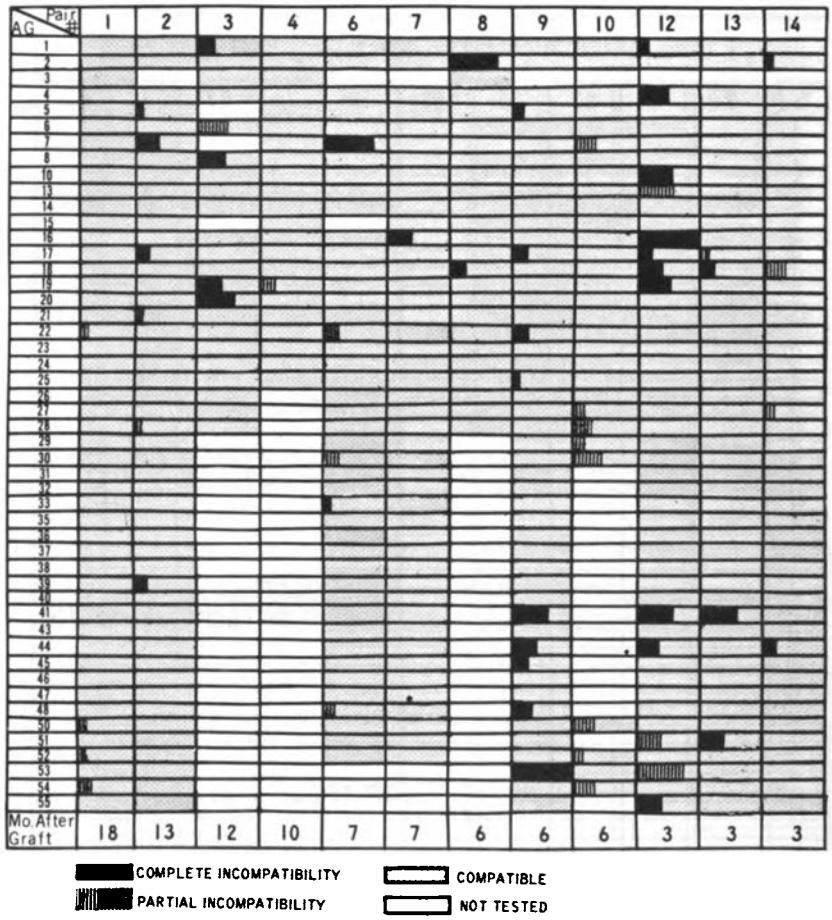

Figure 4.—Summary of the incompatibilities found in kidney homograft survivors. From comparisons of each donor-and-recipient pair as shown in Figs. 2 and 3, the incompatible antigens were determined and plotted. A solid black bar indicates a "complete" incompatibility in which no detectable cytotoxicity was found with recipient cells. A striped bar denotes "partial" incompatibility in which the recipient possesses some antigens, but fewer than the donor. The length of the bars expresses the "units" of difference as defined above ("Results"); a full bar is equivalent to a 5-unit difference.

but insufficient to act as a cytotoxinogen or leukoagglutinogen site. Alternatively, the absorption results may be misleading, for antibodies of varying specificities could be fixed nonspecifically. Pirofsky, Cordova, and Imel (1962) demonstrated that anti-D and normal human globulins fix in equal numbers upon erythrocyte surfaces independently of the antigenic nature of the cell. Nonimmunologic absorption of normal globulins, however, did not lead to agglutination with rabbit anti-human globulin serum. Hence it can be speculated that absorption of antisera may be *less* specific than absorption plus a second reaction (such as antiglobulin absorption or complement fixation).

The premise that quantitative differences in level of reactivity may expose *qualitative* disparities is based on the following reasoning. In any mixed antiserum, not all antibodies would be expected to occur in equal concentrations or reactivity. Antibodies against the stronger antigens may be the most active, and others distributed normally around them. Upon dilution, the mixed antibodies are cut off at different levels, so that at the highest dilution, only the strongest anti-

TABLE 3

Number of incompatibilities of 16 hypothetical donors compared with those obtained with the actual donors. The possible donors were classified according to the total *number* of sera which showed incompatibilities. Complete incompatibilities are defined as those in which the recipient possessed no detectable cytotoxinogens. Partial incompatibilities are those in which the recipient possessed *fewer* antigens than the donor. Numbers correspond to donors (D) and recipients listed in Table 1. • = Actual donor used.

Recipient Number	*NUMBER OF COMPLETE INCOMPATIBILITIES*								
	0–2	*3–5*	*6–8*	*9–11*	*12–14*	*15–17*	*18–20*	*21–23*	*24–26*
# 1	7D 1D•		10D, 7 10 15	2D, 6 15D 6D, 2	13D 9D 9	14D 13			14
# 2	7D 7 6	15D 10D 1, 1D 5, 6D 2D• 10	9 13D	13	14D 9D	14			
# 6	7D 10, 7 10D	1, 2 6D• 2D	15 13D 15D	9 1D 13	9D 14D		14		
# 7	7D•	10D 6, 6D 2, 1 1D	10 13D	2D 15D 15, 9 9D, 10		14D			14
Recipient Number	*NUMBER OF PARTIAL INCOMPATIBILITIES*								
	0–2	*3–5*	*6–8*	*9–11*	*12–14*	*15–17*	*18–20*	*21–23*	*24–26*
# 1	7D	6D 1D• 7	9D 6 15D	13D 2D, 10 2, 14D 13	15 10D 14 9				
# 2	7, 7D 6, 6D 9D, 1 2D•	1D 15D 10 13D	14D 10D 13, 9 15		14				
# 6	1, 7D 9, 7 6D• 9D	14D 15D	2D 13D	10 13, 2 10D 15	9	14			
# 7	7D• 6D	9D, 1 1D, 6 15D	2 14D 13 2D	13D 10 9 15 10D	14				

bodies may remain, since the weaker ones have been diluted to subthreshold levels. Taking the difference in reactivity of the donor and recipient as immunogenic factors, it was shown here that in four long-term survivors, the actual donor used was among the best donors out of 16 hypothetical donors (Table 4). Although this result may be attributable to inadequate numbers of patients tested thus far, it is a surprisingly encouraging result, for this provisional method of adding incompatibilities does not take into account the possibilities that (1) some antisera may have identical specificities, (2) certain antigens may be more potent transplantation antigens than others, and (3)

TABLE 4

Units of incompatibilities of 16 hypothetical donors compared with those obtained with the actual donors. Classification is according to the *units* of mismatch. Unit of difference = difference in number of dilution tubes (1:3) which produced equivalent degrees of cytotoxicity. Complete incompatibilities are defined as those in which the recipient possesses no detectable cytotoxinogens. Partial incompatibilities are those in which the recipient possessed *fewer* antigens than the donor. Numbers correspond to donors (D) and recipients used in Table 1. • = Actual donor used.

Recipient Number	*UNITS OF COMPLETE INCOMPATIBILITIES*								
	0–3	*4–6*	*7–10*	*11–15*	*16–20*	*21–25*	*26–30*	*31–40*	*41–56*
# 1	7D 1D•			2D 15D	6D, 7 6, 10D 10	2, 9D 15	14D	13D, 9 13	14
# 2	7D, 6 15D, 7	1 2D• 10D 1D, 15 6D	10		13D	9D 9 13 14D		14	
# 6	7D 6D• 7, 2	10, 1 10D 2D	15D 1D 15		13D 13	9D 14D 9		14	
# 7	7D•	6 2 6D	10D 10 2D 15D	15	13D 9D	9 13		14D	14
Recipient Number	*UNITS OF PARTIAL INCOMPATIBILITIES*								
	0–3	*4–6*	*7–10*	*11–15*	*16–20*	*21–25*	*26–30*	*31–40*	*41–56*
# 1	7D 1D•		6D 2D 9D 7	10 2, 6 13D 15D 13	14D 10D	15 14	9		
# 2	7, 7D 2D• 6, 6D 10	15D 1, 1D 13D 9D	10D 14D 13 15	9 14					
# 6	7D, 1 1D, 7	9D 6D• 2D	15D 2, 13D 10 14D	15 10D 13 9		14			
# 7	7D•	1D 6D 1, 6	9D 15D 2D 2	14D 15 13 10	13D 10D 9	14 •			

antibodies of numerous specificities may be acting synergistically (Möller and Möller, 1962; Becker and Terasaki, submitted for publication) by complex interactions at each dilution level.

Despite these current problems, certain long-term advantages of a serological approach to typing over other proposed methods can be noted. One important factor is that it does not depend on the physiological functioning of the lymphocyte, as does the lymphocyte-transfer test of Brent and Medawar (1963) and the tissue-culture reactivity tests of Bain, Vas, and Lowenstein (1964) and Bach and

Hirschhorn (1964). Lymphocytes of uremic patients were shown by Bridges and co-workers (1964) to be less capable of giving an intracutaneous reaction than lymphocytes of normal individuals. Too, since physiological activity is imperative for the transfer and tissue-culture tests, the conditions under which the cells are isolated and used are more exacting than in serological methods. The time taken for the reactions to develop precludes the use of nonliving donors (which certainly must be considered the ultimate source of donors). The distinct advantage of sero-typing in being able to mass-type a large battery of donors and recipients at any time and use them in any combination, as is done with blood transfusions, is evident.

The particular serological reaction employed, that of lymphocyte cytotoxicity, appears to have several advantages over the other kinds of reactions previously tested. The method does not suffer from the troublesome difficulties of non-specific agglutination found with leukoagglutination tests (van Rood, van Leeuwen, and Bosch, 1962; Dausset, 1962). The present microdroplet technique permits performance of numerous tests with extremely small quantities of rare human antisera. Moreover, since lymphocytes are often difficult to obtain in large numbers from patients undergoing immunosuppressive therapy, a microtest such as that described is indispensable. For example, from 10 ml of the blood of a kidney-graft recipient, the yield of purified lymphocytes has often been on the order of 400,000 cells, from which 200 separate droplets can be tested in a typical antibody-titration experiment. Nevertheless, for routine large-scale typing, a more quantitative and rapid method of assaying cytotoxicity on an equivalent microlevel would be more desirable. A microcomplement-fixation test was not as sensitive in our hands and entailed the unnecessary introduction of an extra step, as does globulin consumption. Lymphoagglutination, which circumvents the introduction of complement to the antibody-antigen mixture, was found to be less sensitive than the cytotoxicity test by a factor of 10 or more.

The lymphotoxicity reaction was found to be of potential value in avoiding early renal homotransplant failure. In one patient who suffered an "immediate rejection," cytotoxins against lymphocytes of the kidney donor were shown in a sample of serum obtained *before* grafting. Pre-existence of humoral factors has been suspected as being the cause of an immediate reaction by Hamburger *et al.* (1962), but no direct test of pregraft serum with the donor cells was performed. Although the possible pre-existence of antibodies against transplantation antigens was considered by Dempster (1963) in his recent analysis of the anuria phenomenon, it was dismissed as unlikely. From Dempster's review, it appears that as many as 50 percent of clinical renal transplants are anuric for more than 48 hr, though such a high incidence does not occur with canine renal transplants. A point of particular difference between the "first set" reaction of humans and dogs which is not considered by Dempster is the fact that, in contrast to dogs, most uremic patients have usually been transfused prior to grafting. Blood transfusions could lead to formation of antibodies again leukocytes which could immediately cross-react with kidney cells (Terasaki and McClelland, 1963). The "cross match" test in which the serum of the prospective recipient is tested with the donor's cells *before* transplantation is done easily and may be important in preventing a certain number of early rejections. This consideration, however, does not detract from the importance of technical factors, such as minimal ischemia time, which have been shown to play a major role in securing immediate renal function (Starzl *et al.*, 1964b).

With respect to the role of the ABO red-cell antigens, it appears that the A and B incompatibilities should be avoided in general (Starzl *et al.*, 1964a). However, that A and B antigens function as transplantation antigens, or are present on vital sites of a kidney graft, has not been established. Some reason to suspect the above conjectures is the finding that a kidney from a type B person survived in a type A recipient (Starzl *et al.*, 1964a) and is still providing normal renal function after 17 months.

Whether the "minor" erythrocyte antigens are at least of secondary importance has not yet been settled. Woodruff and Allen have reported (1953) an instance in which, in spite of complete matching for 10 antigens, exchange skin homografts were rejected within 3 weeks. On the other hand, in an interesting case described by Peer and co-workers (1960), in which a mother-to-son skin homograft survived for 238 days, only K was incompatible out of 13 antigens tested. A brother's graft also survived for 56 days; the only incompatibility was with Fy^a. Further indication for the influence

of "minor" erythrocyte groups in transplantation is given by Rogers (1963). From the present initial trials, survival for more than 6 months was obtained in spite of incompatibilities with E, Fy^a, M, C, D, s, and c (Fig. 1). Perhaps some significance could be attached to the fact that seven out of 10 long-term survivors (10 to 18 months) possessed two or three antigen incompatibilities. Moreover, in one patient, anti-CD antibodies were found together with good renal function 10 months after grafting. The conclusion which might be indicated is that matching of "minor" erythrocyte antigens may increase the chance of success, though complete matching may not be obligatory.

Summary

A microdroplet lymphocyte-cytotoxicity reaction was tested as a means of predicting histocompatibility matching by titrations of 50 different allogenic human antisera. As the trial system, 11 kidney homograft recipients who have survived 6 to 18 months following transplantation were tested for compatibility with their respective donors. The degree of incompatibility expressed in terms of numbers or units of mismatched antigens was relatively small when compared with that of hypothetical grafts from unrelated donors typed by the same methods. Though many improvements in the typing system remain to be made, it is suggested that with further experience, this method of histocompatibility typing may be practicable in uncovering major transplantation "types."

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Discussion

DR. CEPPELLINI: What is the reproducibility of your test?

DR. TERASAKI: Four persons were typed with 50 different antibodies on three separate days and an average of less than three-tenths of a tube difference was obtained. The percent of living cells at each dilution may vary somewhat from day to day, as with most serological reactions.

DR. METZGAR: I would like to point out that Dr. Terasaki is dealing with 50 antisera, and that one may have a good duplication of the antibodies in these various sera, so that the same antibody could conceivably be found in perhaps 10 sera and would pick out by this method, 10 incompatibilities. In reality, we are really measuring incompatibilities for one antigen. In addition, even when these multispecific antisera react with both the donor and recipient cells they may not be detecting the same antigen, so that by your classification two compatibilities may indeed be incompatibilities. This is the danger of using antisera containing mixtures of antibodies *vs.* monospecific antisera. I would also like you to comment on the rather long-term survivor that had many incompatibilities with the donor lymphocytes by your typing method.

DR. TERASAKI: Your last question goes back to what you said earlier: that it is possible that some of these antisera contain similar antibodies and it's not quite clear what proportion of duplicated antibodies one is dealing with if one counts up the number of these matches by this kind of system. We have only done this as a tentative sort of thing to see if, by doing so, we come out with some sort of order in the long-term kidney-homograft survivors. Certainly, we would feel very strongly that it is much better to end up with pure antibodies than mixed antibodies, and we would like to accomplish this. We have used the method described only as a first trial, rather than spending time trying to purify the antisera, because we did experience difficulty in absorption of antibodies. If it should turn out that each antiserum contains as many as 50 separate antibodies, many years of work would be required to obtain "unit" reagents.

DR. WALFORD: We are also of the persuasion, as I believe Dr. Terasaki to be, that

for serologic typing of human transplantation antigens, the lymphocyte offers a great deal of promise. Rather than using sera of multiparous women, we have investigated lymphocyte cytotoxicity of human sera following actual skin grafts, and after intradermal leukocyte injections. Table 1 shows the titers of such sera in relation to the time of grafting or injection in one of our donor-recipient pairs. All grafts in this table were from this same donor to the same recipient. The donor's lymphocytes were used as the test cell in the cytotoxicity test. The numbers refer to the percent of lymphocytes killed by the serum at each dilution. It is clear that antilymphocyte antibodies were first demonstrable following the second-set graft, and that they increased mildly in strength or avidity with successive grafts. Injection of leukocytes at a much later date (210 days after the fourth-set skin graft) caused a marked increase in the titer of the antibodies. We have also studied these antisera by alcohol fractionation and find that the great majority, and perhaps all, of the antibody activity resides in the gamma$_2$-globulin fraction.

I believe our results may strengthen Dr. Terasaki's very important contribution, for they prove that the lymphocyte cytotoxicity phenomenon which he finds with post-pregnancy serum can also be demonstrated after skin grafting *per se*.

TABLE 1

Lymphocyte Cytotoxicity in Relation to Time of Skin Grafting and Leukocyte Injection. Skin Grafts at Approximately 36-day Intervals; Leukocyte Injection 210 Days After Last Graft.

Serum Specimen	*Titer of Recipient Serum vs. Donor Lymphocytes*					
	1:2	*1:8*	*1:32*	*1:128*	*1:512*	*1:2048*
Pregraft	0	1	—	—	—	—
14 days after 1st set	1	5	—	—	—	—
14 days after 2nd set	43	34	9	—	—	—
15 days after 3rd set	80	50	14	4	—	—
12 days after 4th set	86	70	14	0	—	—
7 days after leukocyte injection	95	61	58	67	51	12

Transplantation Immunity Studies in Man—Bearing Upon Problems in Tissue Typing*

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Application of a skin homograft to a human recipient results in an early period of acceptance during which the graft becomes vascularized and approximates the appearance of the surrounding host skin. Within 6 or 7 days, however, the graft becomes edematous, develops progressive cyanosis, and blood flow in the graft vessels slows and finally ceases (on the 8th to 12th postoperative day). Multiple thromboses ensue and the graft becomes massively hemorrhagic and is replaced by a black eschar. This sequence of events, termed the homograft rejection period (Converse and Rapaport, 1956) is associated with the development in the host of a state of generalized hypersensitivity to subsequent skin homografts obtained from the same donor. The expression of the recipient's sensitivity to grafts from this donor is conditioned by the latent period allowed between rejection of the first-set graft and challenge with a second graft. In the present test system, where 11-mm, circular, full-thickness skin homografts are employed, a latent period of less than 7 days evokes the "white graft reaction," characterized by avascularity and change of the graft into a tan-colored eschar; a latent period of 10 to 30 days, on the other hand, evokes an accelerated, hemorrhagic type of reaction which results in complete graft rejection by the 4th or 5th postoperative day, as compared with the first-set rejection span of 8 to 21 days (Rapaport and Converse, 1957, 1958; Marshall *et al.*, 1962; Henry *et al.*, 1962).

This sequence of events, common to all mammalian species studied thus far (Woodruff, 1960), forms the basis for the contention that rejection of tissue transplants in these species is the result of an immunological mechanism undertaken by the host (Medawar, 1958). Although evidence to support this possibility has accumulated at an impressive rate in the past years, the homograft rejection reaction retains the distinction of being an immunological response in which neither the antigens which evoke the response nor the immune factors which mediate it have been adequately isolated or identified.

It is the purpose of this report to collate a series of studies in human subjects carried out at New York University during the past 15 years. The focus of this study has been a systematic evaluation of the induction, manifestations, and transfer of homograft sensitivity in man.

1. Immunologic Mechanisms of Response to Skin Homografts in Man

A wide array of evidence has been presented to support the possibility that the rejection of skin homografts in various mammalian species is based upon mechanisms similar to those described in hypersensitivity responses of the delayed type (Lawrence, 1959b). There is convincing evidence, as well, that serum antibody may be involved in the rejection of certain types of transplants in animal species (Stetson, 1963). Serum antibody responses have also recently been described in human recipients of homografts (van Rood, van Leeuwen, and Bosch, 1962;

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Colombani and Dausset, 1964); there is, however, no evidence that such antibodies are the actual mediators of graft rejection in man (Kuhns *et al.*, 1964).

In an attempt to study this question, human recipients were sensitized by repeated application of skin homografts obtained from the same donor. At the height of sensitization, leukocytes obtained from the sensitized subjects were disrupted by repeated cycles of freezing and thawing, treated with DNAse, and injected into recipients of grafts obtained from the same source as that used to sensitize the leukocyte donors. Such leukocyte extracts conferred upon the recipients a highly specific state of hypersensitivity to these skin homografts (Lawrence *et al.*, 1960).

It is of interest that (1) serum obtained from sensitized leukocyte donors at the same time as transfer factor failed to transfer accelerated rejection, and (2) transfer factor functioned to cause accelerated rejection both by the systemic and by the local route of transfer.

The latter observation is of particular pertinence to recent suggestions that the results of cell transfers of homograft sensitivity in these subjects could be explained in terms of active sensitization of the recipients by the cell extract (Stetson, 1963). In the course of local transfers of homograft sensitivity leukocyte extract directed against a certain individual's skin (e.g., donor A), was injected intradermally around skin homografts from donor A, and around grafts from a control donor, which had been applied to the recipient 3 days previously. At this time, the grafts were pink and exhibited active blood flow in their surface vessels. Within 18 hr after infiltration of the graft areas with transfer factor, the test graft became hemorrhagic and fulfilled all criteria of accelerated rejection (Kuhns *et al.*, 1964). The control graft, on the other hand, was not affected; it survived for the usual first-set period. It is difficult to attribute this prompt result to active sensitization of the recipient by the transferred materials. Two additional controls lend further support to the interpretation that an actual transfer of immunological information was accomplished: (1) leukocytes obtained from non-sensitized donors failed to cause accelerated rejection of skin homografts, and (2) leukocytes obtained from the same sensitized subject 11 days after rejection of the last sensitizing homograft had lost the ability to transfer homograft sensitivity.

The transfer of homograft sensitivity with leukocyte extract constitutes an important link between the events of skin homograft rejection in man and those observed in hypersensitivity reactions of the delayed type (Lawrence, 1959a). The transfer of homograft sensitivity by this technique provides an effector mechanism for further immunological analysis of homograft rejection reactions in man. The recent demonstration that transfer factor is dialyzable would appear to be of particular pertinence to this problem.

2. Human Tissue Transplantation Antigens

The observation that frozen-and-thawed, DNAse-treated leukocyte extracts retained the ability to act as potent antigens in the induction of homograft sensitivity suggested that leukocytes constituted a ready source of materials for sustained attempts at isolation of the human tissue transplantation antigens (Rapaport, Lawrence, Thomas, and Converse, 1962). In the course of such studies, leukocytes were isolated from peripheral blood, and suspended in 0.25 M sucrose. They were then disrupted by mechanical means in the cold, and their various components were separated by (1) differential centrifugation (Rapaport *et al*, 1963), and (2) dialysis. Nuclear, mitochondrial, and microsomal fractions, fractions rich in endoplasmic reticulum, and two soluble particle-free cytoplasmic fractions, were prepared and tested for their biologic activity as transplantation antigens. Fractions which sensitized the recipients to skin grafts from the same donor were scored as containing transplantation antigens. Table 1 illustrates the results observed.

Cytoplasmic fractions prepared in this fashion regularly induced in the recipients a state of altered reactivity to skin homografts obtained from the leukocyte donors. Skin homografts applied to the recipients 2 weeks after intradermal sensitization with these materials were rejected either in an accelerated fashion (4 to 5 days) or as white grafts. Treatment of the microsomal fraction with RNAse resulted in loss of the ability of this fraction to induce homograft sensitivity. Neither the dialysate of leukocyte extract nor the particle-free dialysis-bag residue had the ability to induce accelerated graft rejection.

Table 2 outlines differences noted between the human transplantation antigens and

TABLE 1
Summary of Results of Sensitization of Human Recipients with Various Tissue-Transplantation Antigen Test Fractions Obtained from Blood Leukocytes

Fraction Used	*Number of Recipients (total:27)*	*Behavior of Skin Homografts in the Recipients*			*Interpretation of the Results (Potency as a Transplantation Antigen)*
		First-Set Grafts	*Accelerated Rejection*	*White Grafts*	
Nuclear	7	4	3	0	Irregular Potency
Mitochondrial	4	0	1	3	Highly Potent
Microsomal	4	0	1	3	Highly Potent
Microsomal After RNAse Treatment	2	2	0	0	Inactive
Endoplasmic Reticulum	6	0	2	4	Highly Potent
Soluble Fraction MW $>$ 40,000	2	2	0	0	Inactive
Soluble Fraction MW $<$ 40,000 (Dialysate)	2	2	0	0	Inactive

TABLE 2
Contrast Between Transfer Factor and Transplantation Antigens Present in Human Peripheral Blood Leukocytes

Transfer Factor	*Transplantation Antigens*
1. Soluble	1. Particulate
2. Dialyzable	2. Nondialyzable
3. Lyophilizable	3. Lyophilizable
4. Resists DNAse	4. Resists DNAse
5. Resists RNAse	5. (?) Labile to RNAse
6. Not a transplantation antigen	6. A potent transplantation antigen
7. Transfers homograft sensitivity	7. Induces homograft sensitivity

transfer factors; it is suggested that these properties permit separation of transplantation antigens and transfer factor present in the same cells, so as to yield a soluble, dialyzable transfer factor on the one hand, and an insoluable, particulate, nondialyzable aliquot of transplantation antigens on the other (Rapaport, Dausset, Converse, and Lawrence, 1964).

The transplantation antigens isolated by this technique have the ability to detect, as well as to induce, sensitivity to skin homografts in man. For this purpose, the microsomal fraction has been the most useful, because of the absence of nonspecific inflammatory reactions resulting from intradermal testing of normal subjects, in contrast with the vigorous delayed reactions it elicits in recipients sensitized to skin homografts from the same donor. The ability of this fraction to resist freezing and thawing and lyophilization (Rapaport, Lawrence, Converse, and Mulholland, 1964) provides additional advantages to the use of microsomes as a stable skin-test material for the detection of homograft sensitivity in man.

3. Tissue Typing in Man

The observation that sensitization to skin homografts from one individual may induce cross-reactivity of the recipient to skin grafts obtained from other donors has raised the possibility that unrelated human subjects may share transplantation antigens (Rapaport, Thomas, Converse, and Lawrence,

1960, 1961; Rapaport, Lawrence, Thomas, Converse, Tillett, and Mulholland, 1962). Implications of this finding have been confirmed and extended by Kuss and Legrain (1961), Mathé (1962), and Wilson, Henry, and Merrill (1963). They form the basis for the "third man test," where compatibility between prospective donor-recipient pairs is tested by the incidence of cross-reactions induced between these subjects' skin grafts, when placed upon a third recipient. Progress in tissue typing based upon this technique has been difficult, because of the cumbersomeness of the method, as well as the occasional equivocal nature of the results obtained.

An alternate approach to the problem of *in vivo* tissue typing in man has been suggested by Brent and Medawar (1963), and has been applied recently to human subjects by Gray and Russell (1963). This method is based upon the induction of a graft-*vs.*-host reaction in blood lymphocytes from one individual, injected intradermally into a panel of potential graft donors. Those subjects whose skin reactions are the smallest are then considered to be the ones most compatible with the future recipient.

The first portion of this report has described the isolation of an effector mechanism concerned with the rejection of skin homografts in man, and preparation of a relatively stable subcellular extract containing human skin transplantation antigens. These materials may permit application to the problem of histocompatibility typing in man of certain recent observations made with the cell-transfer system in delayed bacterial and fungal hypersensitivity.

In the course of such studies, intradermal injection of mixtures of transfer factor plus the specific antigen against which it was directed have been noted to elicit vigorous delayed skin reactions in the recipient. The reactions were specific, and did not occur unless the proper antigen was added to the solution of transfer factor (Rapaport, Lawrence, Miller, Pappagianis, and Smith, 1960). The high degree of individual specificity observed in the transfer of homograft sensitivity in man may make such an approach particularly applicable to problems of *in vivo* skin tests of histocompatibility in man (Rapaport, Lawrence, Thomas, and Converse, 1962). It suggests a system of intradermal histocompatibility testing which may eliminate possible variables resulting from differences in the hosts' immunologic status, as it is primarily dependent upon local interaction between transfer factor and antigen.

It must be emphasized that it is by no means certain that tissue types exist in man. Grave questions have been raised on this subject, particularly in view of the uniform rejection of all skin homografts performed between unrelated or even related individuals, with the exception of those exchanged between identical twins. The incidence of cross-reactions noted in studies of homograft sensitivity in man has been used as a potent argument to suggest the existence of tissue types (Wilson, Henry, and Merrill, 1963). It may rather have been an expression of the state of hypersensitization of the recipients, with an associated blunting of the specificity of their responses. (Rapaport, Lawrence, Thomas, Converse, Tillett, and Mulholland, 1962). The technique for histocompatibility testing suggested here may, however, provide some further clarification of this question. It is possible that the antigenic determinants which induce transplantation sensitivity in man may not be amenable to classification into groups which would provide a significant degree of donor-recipient compatibility. In this case, the effector mechanism and preparation of human transplantation antigens described in this report may serve as a point of departure for the further application of more precise immunological techniques to this problem in man.

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Discussion

DR. RUSSELL: Would you like to say anything about the relationship between red-cell antigens and transplantation compatibility?

DR. RAPAPORT: As far as this relationship is concerned, we have tried very extensively, in collaboration with Dr. W. J. Kuhns, to find some kind of relationship between red-cell antigens, using all of the known groups and subgroups, and transplant compatibility, using the skin-homograft system as endpoint. So far, we can find no significant correlation. We have also approached this problem by trying to sensitize human recipients to skin homografts with red cells and red-cell membranes, but so far have no evidence that there is any sensitization to skin homografts in the recipients.

DR. van ROOD: Apparently you get only monospecific sensitivity by the use of transfer factor; in other words, the recipients do not show sensitization with other random people. Might this be due to dosage, that is, if you use more transfer factor, might you have some reactions with random people?

DR. RAPAPORT: This certainly is a possibility. I would suspect that the more a

given individual is hypersensitized, the less individual-specific will his response become, regardless of whether you provide him with effector mechanisms or with transplantation antigens.

DR. LALEZARI: Could you go a little further in talking about the distribution of these antigens amongst various leukocyte cells. We are all talking about leukocytes as a whole. I would like very much to know the distribution of these antigens.

DR. RAPAPORT: The blood used in these studies is collected through a Fenwal bag with a cation-exchange resin chamber which takes out calcium and removes about 90 percent of the platelets. We have seen no platelets in our final leukocyte suspension smears. The differential of the final preparation prior to mechanical disruption is approximately 50/50 mononuclear cells/granulocytes.

DR. BRENT: I'd just like to question, for a moment, the last two lines on your table comparing transfer factor on the one hand and extracted antigen on the other. If I remember rightly, you said the table stated that transfer factor does not sensitize actively but that antigens do. But in fact, the design of your experiment in which you tested the potency of either of these two preparations was, in the course of time, the same. In other words, the interval between injection of transfer factor and antigen and the application of the skin graft was about 2 weeks. If that is so, how can you, in fact, distinguish between active sensitization and transfer of an antibody-like fraction?

DR. RAPAPORT: The experimental design in the transfer of sensitivity to skin homografts was actually quite different from that employed in the active induction of homograft sensitivity with antigen fractions. In the first instance, skin from subject "A" was used to hypersensitize subject "B," whose leukocytes were now used to *transfer* sensitivity to "A" grafts in a third subject, "C." Such transfers of sensitivity could be accomplished either locally, by infiltrating the test grafts with anti-"A" transfer factor, with rejection of the grafts within 18 hr, or systemically, by pretreating subject "C" with anti-"A" transfer factor. This is quite different from the studies of the induction of sensitivity to skin homografts with antigen fractions isolated from leukocytes. In the latter instance, *instead* of using skin homografts from subject "A" to sensitize subject "B," we used leukocyte fractions injected intradermally, in an attempt to ascertain which fraction of the leukocytes of subject "A" could have an effect in subject "B" similar to that observed after a skin homograft from subject "A."

DR. MILGROM: Can you transfer "A" antigen with "B" leukocytes? In other words, could "B" leukocytes carry along some "A" antigen?

DR. RAPAPORT: It is certainly possible that, if by some chance selection, subjects "A" and "B" in the transfer experiments happen to share some transplantation antigens, then the recipient of anti-"A" leukocytes obtained from subject "B" might be actively sensitized to "A" skin grafts. I would like to point out, however, that the survival times of "A" grafts on subject "B" did not show *any* evidence of such compatibility (i.e., antigens in common) between subjects "A" and "B." Furthermore, in individuals who received systemic transfers of sensitivity (where the incubation period used was the same as that employed in the active sensitization studies), the transfers were individual-specific in each instance, and the success of transfer depended upon the time at which the hypersensitized donor of transfer factor was bled. One experiment designed to study this question specifically was as follows: blood was taken from subject "B" at the height of his sensitization to skin homografts from subject "A"; transfer factor prepared from these cells successfully transferred sensitivity to skin homografts from subject "A" applied to subject "C" in every instance. When, however, an additional aliquot of blood was removed from subject "B" 11 days after the first bleeding, transfer factor prepared in the same fashion from this blood had *lost* the ability to transfer sensitivity to "A" grafts, which were accorded a normal first-set survival time in subject "C."

Additional observations which substantiate the contention that the observations made with transfer factor were the result of an actual transfer of immunological information, and *not* of cross-sensitization, are based upon the local transfer of homograft sensitivity. In this instance, transfer factor prepared from blood obtained from the suitably sensitized subject "B" was infiltrated around test grafts (or "A" grafts) and control homografts, which were in their 3rd day of residence on the arm of subject "C." In every instance, anti-"A" transfer factor infiltrated around a graft from donor "A" caused the accelerated rejection of this graft

within 18 hr. Anti-"A" transfer factor had no effect upon grafts from other donors, and normal leukocyte extract obtained from nonsensitized donors did not affect the survival of "A" grafts.

It would be difficult to ascribe such an observation to a mechanism other than the actual transfer of specific immunologic information to the recipient.

DR. CEPPELLINI: I would like to know how often there was activity of the transfer factor on the third party.

DR. RAPAPORT: Every time we used transfer factor obtained from an individual who was adequately sensitized—the adequacy of sensitization required four successive accelerated rejections of subject "A" skin—this transfer factor could transfer sensitivity.

It may be of interest that, when we tried to transfer sensitivity to homografts by using leukocytes obtained from an individual who had been sensitized with four successive white-graft-rejection reactions, we would not transfer sensitivity with either serum or leukocytes.

DR. CEPPELLINI: Many people, including I think yourself, have shown that very often there is a cross-sharing of antigens, and in, for instance, a white graft, you use it against many other people. Now, if, by chance, subject "C" shared the same antigens as "A," anything, perhaps the transfer factor antibody, should be absorbed on the tissue of "C" and therefore you get a second incidence of failure.

DR. RAPAPORT: Yes. To begin with, I'd like to clarify a statement we made some years ago in the Journal of Clinical Investigation about cross-reactions. I think, certainly, one possible interpretation of the cross-reactions observed in individuals hypersensitized to skin homografts by means of either repeated grafts or leukocytes may be a possible sharing of antigens. It is just as possible, however, that the so-called cross-reactions occurred because of a blunting of the individual's specificity of responses because of this hyperimmunization. Therefore, while antigen sharing is a possibility, it may not necessarily be *the* explanation.

DR. DAUSSET: Do you think that these transfer factors can be used for typing?

DR. RAPAPORT: Well, I would certainly hope so.

DR. SHULMAN: I just wanted to ask a question concerning your thoughts on the relationship of transfer factor to anything else described in the past, biochemically and biologically.

DR. RAPAPORT: I think it would be fatuous on my part to discuss this subject any further, as Dr. Lawrence, who trained me in this area, is present. I wonder if he'd care to answer the question.

DR. LAWRENCE: I think part of the difficulty with our understanding of the nature of transfer factor has been just this inability to relate it to previous experience. As far as we know, immunochemically it is not antibody, it is not a globulin fragment and biologically it behaves like a material that is transferring immunological information. The materials present in the dialysate that are potentially biologically active are polypeptides and polynucleotide chains. I think the most important question, either to reinforce or lay low at this time, is whether or not transfer factor is a type of micromessenger RNA (Lawrence *et al.*, 1963).

DR. SHULMAN: In which case, how would you suspect it works?

DR. LAWRENCE: I would suspect that it works by getting into the cells of the recipient and interacting with materials that are ordinarily concerned with this sort of activity. The possibility also exists that it could be a polypeptide with this sort of biological activity. The one thing certain is that the material transferred is not an antibody. Less certain is the possibility that minute fragments of antigen could be coupled to polynucleotide chains, the way Campbell and Garvey have shown, and persist for prolonged periods of time. I have an open mind in the matter, I hope not too porous, but at least open enough to try to find out what it is.

It is very possible that antigen could be carried over. In local transfer I think this is extremely less likely with an 18-hr latent period. Moreover, in order to do a local transfer, the donor was sensitized by only two graft exposures, with a latent period in between. This minimal sensitization schedule allowed his leukocytes to transfer local but not systemic sensitivity. On one arm of the recipient was a test and a control graft and the other arm had a test and a control graft. Only the locally infiltrated test graft was rejected. In order to get the systemic transfer one needed four exposures to skin

grafts. There are a lot of details in that paper which clarify the particular question you asked and others as well (Lawrence *et al.*, 1960). One of the other aspects of interest in this connection is that one individual who, at the height of his rejection, was able to transfer sensitivity to four recipients then had flareups at all of his old skin-graft sites and 11 days later, when leukocytes were in turn obtained from the same individual, was unable to transfer sensitivity. To us this suggests a desensitization occurring *in vivo* the way Pappenheimer and I had shown it to occur *in vitro*. When this leukocyte donor was challenged again with the skin graft used originally to sensitize, he reacted, as if he had never met it before, with a first-set reaction.

DR. RAPAPORT: One possible approach to the problem of tissue typing, based upon the use of transfer factor and transplant antigens, is as follows: Transfer factor directed against subject "A" would be incubated with antigens from subject "A," and antigens from another nine unrelated subjects. These mixtures would then be incubated at 37°C for 30 min, by a technique similar to that used for mixtures of transfer factor and antigen in other delayed hypersensitivity systems; these mixtures would then be injected intradermally into the arm of an indifferent recipient. The mixture of anti-A transfer factor and "A" tissue transplantation antigen may give a strong reaction in 24 to 48 hr, if the basic analogy to the delayed allergy system is confirmed. Any other transplant antigen source that will give a similar reaction in this individual when mixed with anti-A transfer factor could then be interpreted as coming from individuals possessing antigens similar to those present in subject "A."

DR. CEPPELLINI: Supposing that you have subjects "A" and "C" who are monozygotic twins; they have the same antigens. You immunize "B" against "A," and therefore against "C." What will happen?

DR. RAPAPORT: I suspect that you would probably get hemorrhagic necrosis at the site of injection of the anti-A transfer factor in subject "C."

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The Lymphocyte Transfer Test in Man*

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When organs are transplanted amongst randomly selected animals of ordinary genetic diversity in which the immune mechanism has been suppressed by a standard program of drug treatment, there is considerable variation in their survival time, a proportion functioning for many months (Calne, 1963; Barnes and Flax, 1964). The same pattern obtains in human transplantation, in that on a similar immunosuppressive regimen there is a wide scatter of survival times. We believe that this variability is a reflection of the degree of genetic disparity between donor and recipient. It has been well documented, using inbred strains of mice, that the smaller the histocompatibility differences between donor and recipient, the easier it is to break the immunological barrier to graft acceptance (Snell *et al.*, 1953; Berrian and McKhann, 1960; Billingham, Brent, and Medawar, 1956; Medawar, 1963; Main and Prehn, 1955; McLaren, 1961). That a similar situation exists in the human population is suggested by the published figures from the previous conference in the present series on renal homotransplantation (Murray, 1964), which clearly suggested that more success had been obtained when donor and recipient were closely related. In order to improve the chances of success in human homotransplantation a test that will assist in selection of a relatively compatible donor is required. The argument that histocompatibility matching cannot often be practically helpful has not been valid in our experience, since on several occasions there has been a choice of donor from a number of potential candidates.

A preliminary account by us in 1963 (Gray and Russell, 1963) described a potential screening test for donor selection in human transplantation. These experiments, carried out in human volunteers, were based on the normal lymphocyte-transfer (NLT) test described by Brent and Medawar (1963). We present here a fuller account of the biological events involved in this reaction.

Our aim in this test is to assess the response of the recipient's lymphocytes on contact with donor antigens. To the extent that the inflammatory lesion produced by the intradermal (I.D.) injection of living homologous lymphocytes reflects a graft-*vs.*-host (GVH) reaction, it is a measure of this factor. Evidence bearing upon this question has been gathered. Experiments have been done to determine the onset and time course of heightened specific reactivity of an individual receiving an inoculum of homologous lymphocytes. The sensitivity of the test as a means of selecting the least incompatible donor from a panel was examined by transfering skin grafts from cell recipients to cell donors and attempting to predict, from the skin reactions, the order of rejection of the grafts. In a small number of cases the test has been used in the selection of human kidney donors and an attempt made to correlate their postoperative courses with the results of the test.

Materials and Methods

Subjects. It is important to point out that only cells from normal donors were used in experiments involving volunteers and that the dangers (e.g., of transferring virus diseases) were explained. When uremic cells were used, family members only received them after suitable explanation of the possible dangers, and controls were from patients in the hospital with incurable disease.

Preparation of lymphocytes. Essentially, the method previously described (Gray and Russell, 1963) was used. However, sometimes

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with this technique polymorphonuclear (PMN) contamination was above average; this was especially so in uremic patients, whose lymphocytes formed less than 10 percent of their total white-cell count in most cases. When this occurred further purification was carried out by adding Carbonyl-Iron Powder S.F. 100 mg to 10 ml plasma and gently agitating for 1 hr at 37°C. Differential centrifugation then removed the iron filings and with them the polymorphonuclears which had ingested the iron. Lymphocyte suspensions of 92- to 100-percent purity and over 90-percent viability were obtainable by this method.

Procedure. From 2.5 to 5 $\times$ 10^6 cells in 0.1 ml of serum were injected intracutaneously on the flexor surface of the forearm. The resulting inflammatory lesions were read definitively at 48 hr and scored on a 0 to 4+ basis, dependent on the measured diameter of erythema and induration.

Fitted full-thickness skin grafts measuring 11 mm in diameter were applied to the flexor aspect of the forearm, where they were anchored in place for about 4 days with 5-0 silk sutures. The criteria of rejection were those described by Converse and Rapaport (1956):

(1) cyanosis and marked edema of graft,

(2) thrombosis and extravasation of blood in graft, and

(3) intense secondary erythema of adjacent host skin.

In agreement with Rapaport *et al.* (1962) we found no instance of a first-set skin graft's being rejected by the 6th day, so that rejection of a graft before this time was recorded as accelerated rejection.

Results

Controls. Autologous lymphocytes consistently gave negative reactions at 48 hr, although in some cases an early flare was produced which quickly subsided. Autologous PMN leukocyte and platelet suspensions not infrequently gave rise to marked inflammatory reactions reaching a peak by 24 hr. Because of this it was felt important to eliminate far as possible in order to avoid adding non-immunological inflammatory changes to the reactions. Full blood grouping has been done on all individuals and does not correlate with the reactions.

Homologous lymphocyte reaction. Viable, normal homologous lymphocytes produced inflammatory reactions ranging in size from 5 to 15 mm with the mean being 7 to 9 mm and reaching their maximum intensity between 24 and 48 hr. An early flare reaction, frequently present but fading after 24 hr, made reading of the test at 48 hr advisable. Of some 200 reactions, 75 percent were scored in the 1+ to 2+ range (see Fig. 1).

After reaching its height at 48 hr, the behavior of the lesion varied. The majority maintained maximum intensity for a day or two, then gradually faded, usually disappearing by the 10th day. A number maintained their 48-hr appearance for 7 to 10 days before gradually fading. Normal lymphocytes in the dosage selected never failed to produce reactions. Homologous uremic cells, when injected into blood relatives, produced occasionally no reaction and in general smaller reactions than when injected into nonrelated subjects. In both cases these reactions were generally smaller than those produced by normal lymphocytes. The small number of cases involving uremic cells precluded statistical analyses of the lesion size and of its correlation with the degree of uremia. These findings with uremic cells are in general agreement with the similar observations recently reported by Bridges, Nelson and McGeown (1964). Some 30 percent of lesions, after fading in part or entirely, showed a sudden flareup between the 8th and 10th days. On one occasion when cells killed by repeated freezing and thawing had been injected as a control for viable lymphocytes a recrudescence appeared at the site of the living-cell injection and was accompanied by a flare at the site of the killed cells, which had failed initially to produce any reaction.

There are, then, two phases in this event, an initial reaction which we believe to be a GVH reaction, followed by a secondary reaction which is probably a host-*vs.*-graft (HVG) reaction.

Individual skin reactivity varies considerably and no satisfactory control of this is available. Nevertheless there was some correlation between cell dosage and reaction intensity. With cell dosage below 2 $\times$ 10^6, reactions had a poor correlation with dosage these elements from the test suspensions as and were sometimes negative. From 2 to 5 $\times$ 10^6, the mean grading rose from a 1+ to a 2+ but the scatter was similar; around 5 $\times$ 10^6, more of a plateau was reached (Fig. 2).

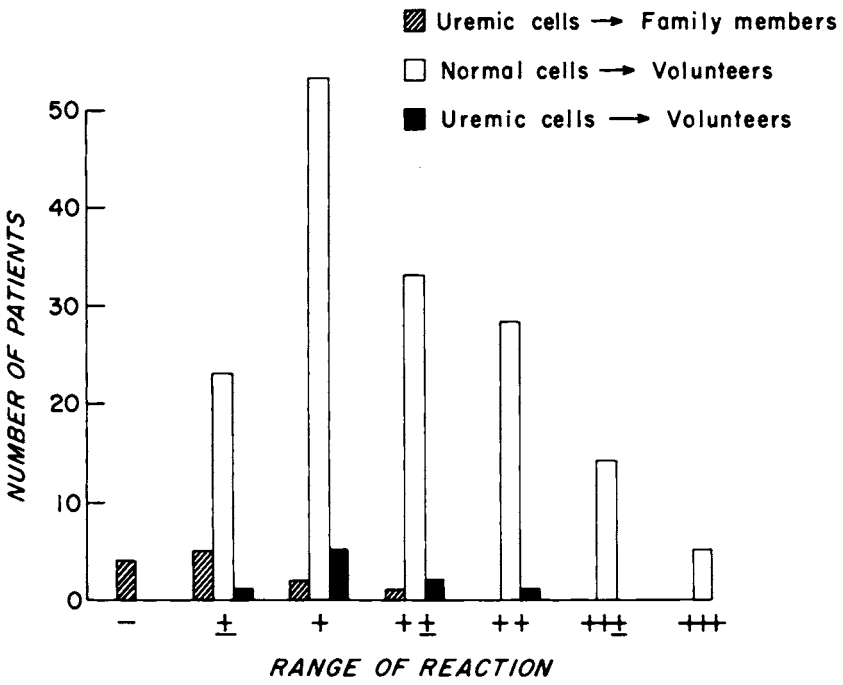

Figure 1.—Histogram of the range of reaction intensities seen in 175 cell transfers. The majority of reactions produced by normal cells fall in the + to ++ range. Uremic cells in general produce smaller reactions than normal cells but these reactions correlate well with the known genetic relationship of donor and recipient.

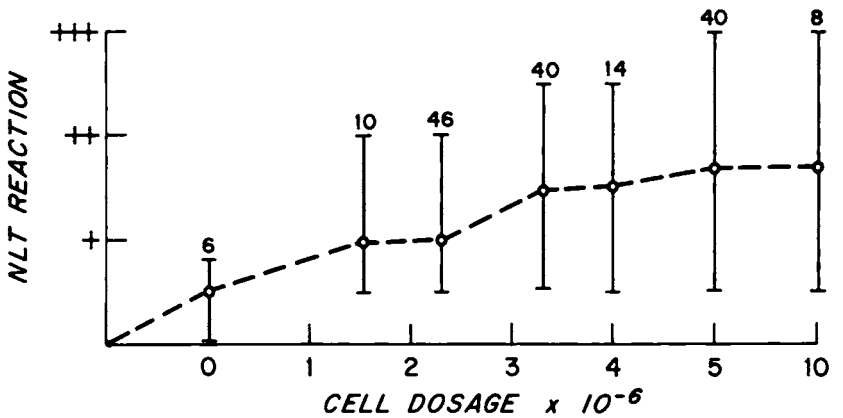

Figure 2.—The relationship of NLT reaction size to cell dosage is charted with the scatter of reactions indicated. Cell dosage below 2×10^6 did not correlate well with the reaction intensity. Going from 2×10^6 to 5×10^6 there was a rise in the mean grading from + to + ±; at the latter dosage a plateau was reached.

Histology. Biopsies were obtained at intervals of from 12 to 72 hr. The 12-hr sections showed a moderate number of mononuclear cells and considerable cell debris, indicating cell death. The later sections showed a marked increase in the number of cells which were aggregated in islands in the deeper layers of the dermis (Fig. 3);

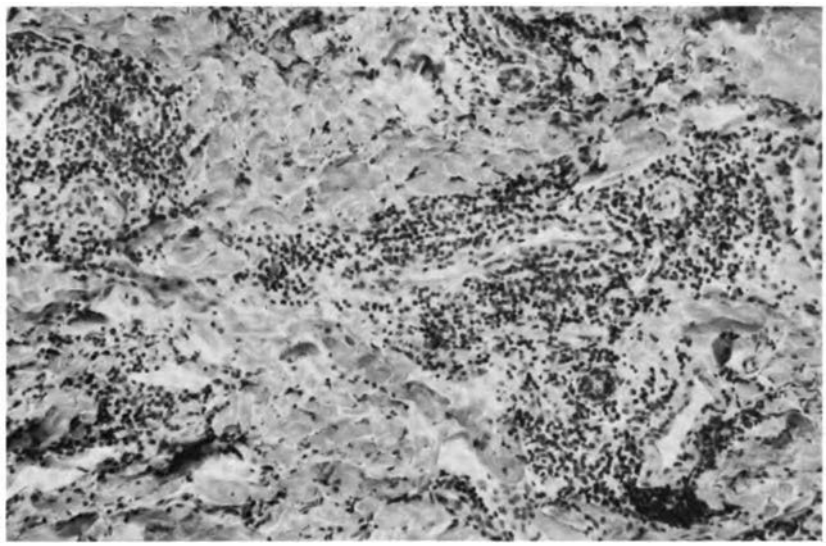

Figure 3.—Histological section of biopsy of skin reaction at 48 hr following transfer of 5×10^6 homologous lymphocytes. Cell islands are seen in the deeper layers of the dermis. (H and E) × 43.

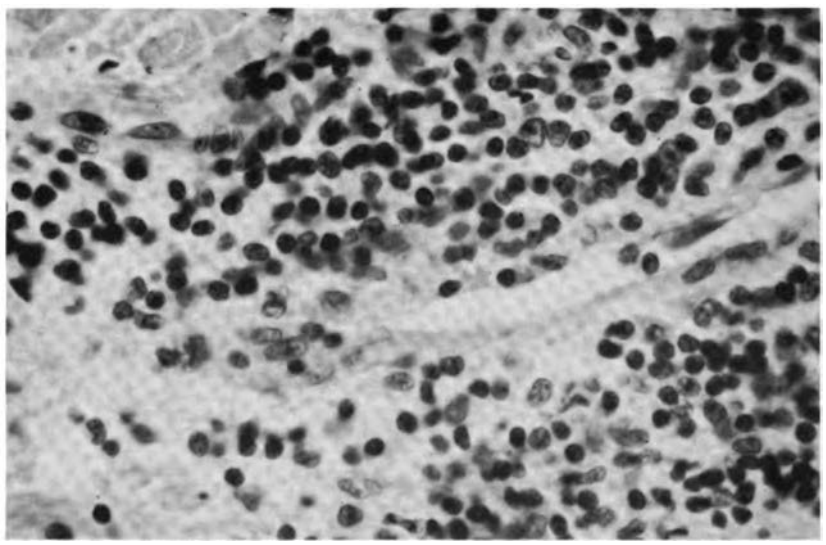

Figure 4.—Histological section of biopsy of skin reaction at 48 hr following transfer of 5×10^6 homologous lymphocytes. The cells are seen to be mainly mononuclear in type, the majority being small lymphocytes. The perivascular arrangement of the cells would suggest that many cells at this stage may have been derived from the host. (H and E) × 98.

these islands were not exclusively perivascular. The cell type involved was predominantly mononuclear, with the small lymphocyte being the commonest (Fig. 4). No mature plasma cells were seen but a moderate number of large pyroninophilic cells were present and a number of mitoses were seen, indicating that local replication was responsible, at least in part, for the increase in the number of cells present.

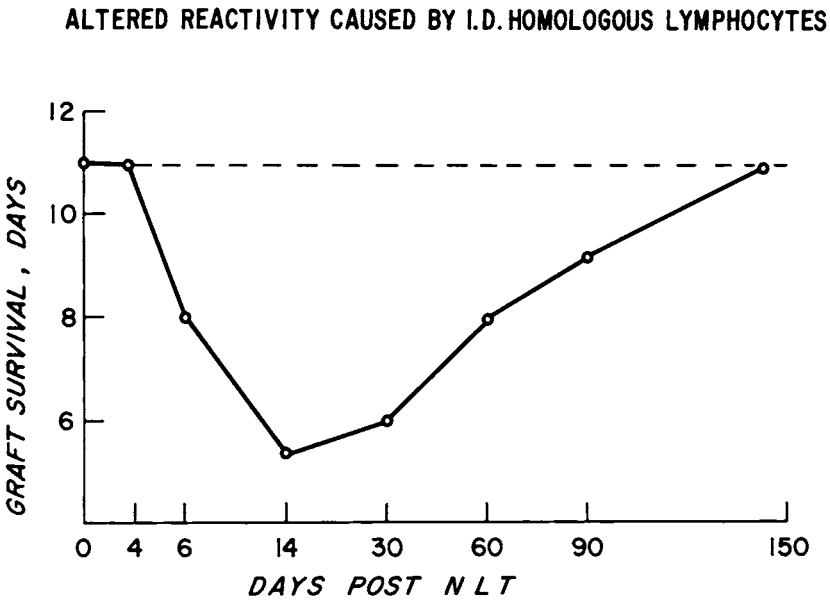

Figure 5.—The sensitization of recipients of I.D. lymphocytes as indicated by the accelerated rejection of specific skin grafts is first apparent at 6 to 8 days, is marked at 14 days, and has disappeared at 5 months.

	A.C. No.1	R.C. No.2	W.Mc. No.3	W.M. No.4	R.K. No.5
D.R.	±	±	+	±	+
C.F.	±	+ ±	+ ±	+	+ +
H.C.	±	±	+ ±	+	+ +
D.F.	+ ±	+ ±	+ ±	+ ±*	+ ±
J.B.	+	+	+ ±	+ ±	+*
E.A.	+ +	+ + ±*	+ +	+ +	+ + +
H.D.	±	+*	+ ±	±	+ +
E.C.	±	+	+ ±	±	+++ ±*
F.W.	+ +	+ +	+ +	±*	+ + +

Figure 6.—Plan of experiment to assess discriminatory powers of test in selecting skin-graft donors. Lymphocytes from donors 1 to 5 were transferred to recipients (vertical column) and the reactions graded. Pairs of cell recipients were then selected as indicated and skin was transferred from them to the cell donor with the prediction that the graft from the stronger reactor would be rejected earlier than that from the weaker reactor. (See Fig. 7).

	N.L.T. GRADING	GRAFT REJECTION (DAYS)
A	+ +++	12 10
B	± ++±	17 12
C	+ ++±	11 11
D	± + ++	9 11 9
E	+ ++	12 12

	N.L.T. GRADING	GRAFT REJECTION (DAYS)
F	+ ++±	11 8
G	+ ++	11 12
H	± ++	16 10
I	+ ++ ++±	11 11 9
J	± + ++±	12 11 11

Figure 7.—The results of experiment described in Fig. 6. It is seen that predictions of the order of skin-graft rejection were correct in nine instances, incorrect in two, and in the remaining five the grafts were rejected on the same day.

Efforts to label the cells with tritiated thymidine and adenosine *in vitro* have so far not been sufficiently successful to employ this method of distinguishing host from graft cells.

Altered host reactivity. The time of initiation of sensitization was estimated by the use of test skin homografts from the lymphocyte donor placed at varying times in relation to the cell injection. Although this maneuver clearly introduces an additional dose of sensitizing antigens, some measure of the state of host reactivity can be appreciated by the state of the skin graft, and a prior state of considerably heightened sensitivity is readily demonstrable as a "white graft" reaction. It is seen from Fig. 5 that some sensitization occurred by 6 to 8 days and is marked by 14 days, at which time a white graft reaction was produced in some instances. Sensitivity was still present at 3 months, but less marked, and had disappeared at 5 months.

In all of these experiments, a marked inflammatory reaction appeared at the site of the lymphocyte injection at the time of skin-graft rejection. The specificity of these experiments is difficult to control because of the common cross-reactivity caused by sharing of histocompatibility antigens in the human population (Friedman *et al.*, 1961). Nevertheless, if a 10-day inflammatory response at the lymphocyte site occurred when the graft and lymphocytes came from differ-

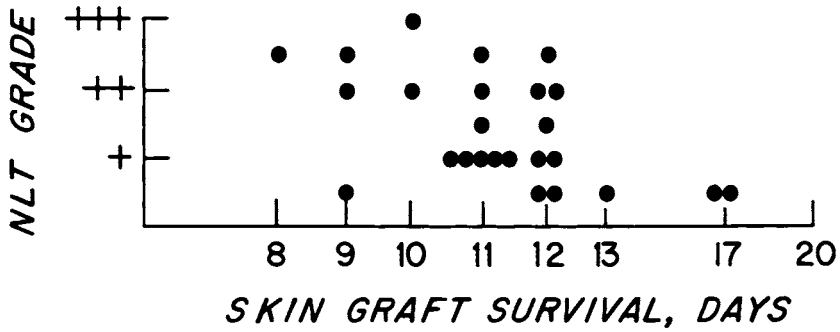

Figure 8.—Skin-graft survival times plotted against lymphocyte reactions. A general correlation between the magnitude of the skin reaction and the survival of retrograde skin homografts is apparent. The unexpected rejection at 9 days of a graft from a ± reactor can be explained on a technical fault. In this instance the cell recipient showed no evidence of sensitization by test with a skin graft of donor origin and thus probably received an inadequate cell dosage.

RECIPIENT	REACTION TO LYMPHOCYTES FROM:	
	PATIENT (A-)	CONTROL (O-)
WIFE (A-)	+	++±
SISTER (O+)	−	++
VOLUNTEER (O+)	±±	++±

Figure 9.—Results are shown of the lymphocyte-transfer test used in selecting a kidney donor. Blood groups are shown in parentheses and appear to be unrelated to the intensity of the lymphocyte reaction.

ent sources, it was never as intense as when they were isologous. Also, with grafts placed after sensitization by I.D. lymphocytes, the specific skin was always rejected first, though on frequent occasions the nonspecific skin showed evidence of accelerated rejection.

By kind permission of Dr. K. Frank Austen we had the opportunity of carrying out the test in a patient with ataxia-telangiectasia (Reye, 1960). This hereditary disease is characterized by a progressive cerebellar deficit, oculocutaneous telangiectasia, and varying serum gamma globulin deficiencies. In this particular case, the total lymphocyte count was 28 percent of normal and gamma 1A globulin was absent from the serum. The patient was found to be incapable of becoming sensitized by the topical administration of dinitrochlorobenzene (DNCB) and rejected three homologous skin grafts from randomly selected normal donors in approximately 35 days. The injection of homologous lymphocytes into normal donors, however, produced reactions which were within normal limits.

Discriminating ability. Experiments to determine the discriminatory ability of the normal lymphocyte-transfer reaction to se-

lect the least incompatible donor from a panel were set up in a checkerboard manner (Fig. 6). Each member of a panel was injected with lymphocytes from several sources. The reactions produced by lymphocytes A in numbers 1 through 9 were compared; skin was then taken from a pair of cell recipients who showed a difference in NLT reaction and placed on the cell donor with the prediction that the skin graft from the individual showing the greater reaction would be rejected before that from the individual showing the lesser reaction.

The results are shown in Fig. 7. On examining each pair, it can be seen that in nine cases the prediction was correct, in two cases the order was the reverse from that predicted, and in five cases the grafts were rejected simultaneously. In comparing the skin graft survival time with the NLT intensity, it is apparent from Fig. 8 that there is some correlation between them. The unexpected early rejection at 9 days of a graft from a ± reactor can be explained by a technical error, for on subsequent testing for sensitivity by skin graft from the cell donor no accelerated reaction was found and it is therefore doubtful whether an adequate dosage of cells had been given.

Patients. In five consecutive instances the test has been carried out with the cells of patients requiring renal homotransplantation. The result of one of the tests is shown in Fig. 9. One of the patients has not yet been operated upon, and a second patient died in the immediate postoperative period of a myocardial infarction. In a sixth case the test was only done retrospectively some 5 months after transplantation, at which time the test first became available. Although many factors are of great importance in selecting an appropriate kidney donor, we have given considerable weight to the question of histocompatibility and, in the interests of brevity, will not detail other considerations here.

Bilateral nephrectomy is performed on the recipient in all instances at the time of transplantation. In patient A, a 7½-year-old girl with renal hypoplasia, we selected the father rather than the mother as donor with the help of the test. This patient has had a remarkably uneventful recovery with no rejection crises having occurred during the 8-week period since the operation. Her renal function is entirely normal.

Patient K, with terminal renal failure from chronic glomerulonephritis, received a kidney from his sister who was selected as kidney donor with the aid of the test. He had good renal function from the beginning but developed a low-grade fever, probably due to a mild rejection process during the 3rd week postoperatively which readily responded to actinomycin C and prednisone. He has now returned to full work and continues to have perfectly normal renal function at 5 months.

Patient L, with terminal renal failure from renal calcinosis, received a kidney from his brother in whom he had produced a ± reaction. He, however, had a course marked by two very distinct episodes of rejection, the first beginning on the 4th postoperative day. If he had been sensitized by prior blood transfusions, of which he had had many, which might have shared histocompatibility antigens with his brother's kidney, this situation was not revealed by the lymphocyte test. This patient now has normal renal function 9 weeks following transplantation.

The patient in whom the test was done retrospectively is now at home doing a full day's work with normal renal function some 15 months after transplantation. It was of interest to note that in spite of 3 months of treatment with Imuran his cells were fully capable of producing strong reactions in several unrelated controls whilst in his donor his cells produced a much smaller reaction than did control cells. His response was normal when stimulated *in vitro* with phytohemagglutinin (PHA) (Bach and Hirschhorn, 1964).

Discussion

For the normal lymphocyte-transfer test to be of value in selecting a tissue donor, the visible lesion must be on the basis of a reaction of graft against host; i.e., the reaction must be the result of immunological activity by the injected lymphocytes stimulated by the cell recipients' antigens. Clearly the actual induration and erythema are the result of host participation but their intensity, we believe, is heavily influenced by specific immune activity on the part of the inoculum of foreign cells. That unsensitized cells should, on meeting a new antigen, react in 24 to 48 hr may be surprising, but Gowans (1962) has shown that when thoracic duct lymphocytes are infused into F_1 hybrids to produce a classical GVH reaction, large pyroninophilic cells demonstrably of donor origin are seen in the host spleen within 12 to 24 hr. It seems probable that some, and perhaps most, of the mononuclear cells seen

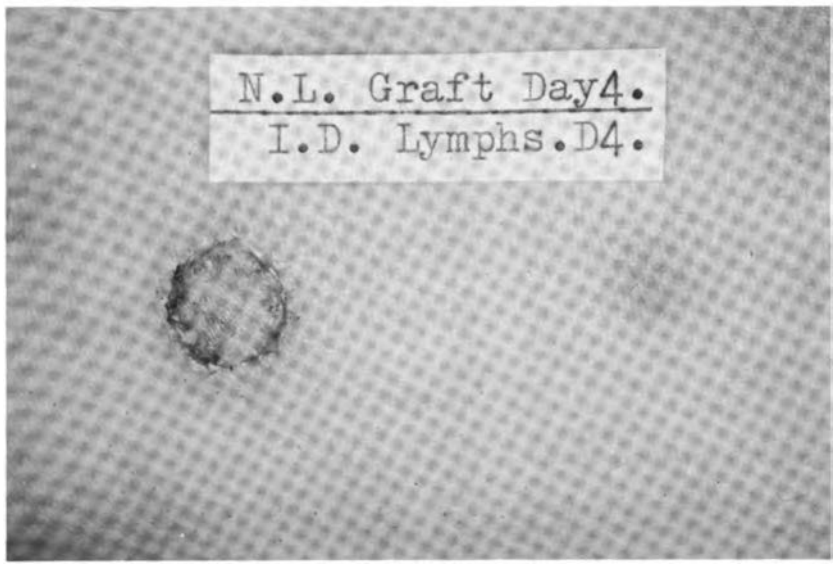

Figure 10a.—Isologous skin and lymphocytes were transferred simultaneously to a homologous host. At day 4 the graft is healthy and the lymphocyte reaction is fading.

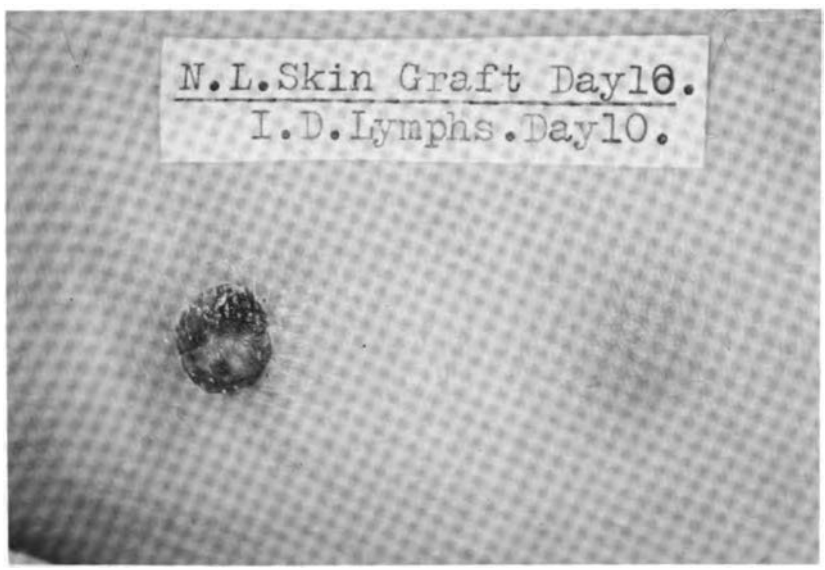

Figure 10b.—The same recipient shows at day 10 rejection of the skin graft and a marked increase in the inflammatory reaction at the site of the injected lymphocytes. This indicates that the secondary phase of the lymphocyte reaction is HVG in nature and also that host sensitization by the I.D. lymphocytes does not occur until after the primary phase has passed.

in the skin reactions described here are host in origin, as it is unlikely that replication of the injected cells could account for the numbers found. This, however, is in keeping with studies of the origin of cells involved in other GVH reactions (Simonsen, 1962), including the transfer reaction in rabbits (Kosunen and Dvorak, 1963), and

in delayed hypersensitivity reactions following the cellular transfer of sensitivity (McCluskey, Benacerraf, and McCluskey, 1963).

If the initial 48-hr reaction were HVG, then host sensitization would have occurred and a subsequent skin graft from the cell donor should be rejected in an accelerated fashion. In practice, evidence of sensitization is not detectable by this means until 6 to 8 days after the lymphocyte transfer. Furthermore, if the initial reaction was HVG, it would be difficult to explain the recrudescent phase seen at 10 days. The difference in direction of these two reactions at 2 and 10 days is illustrated by the fact that frequently the pattern of the two is altered; that is, the order of intensity of reactions produced by cells from a number of sources was not the same at 10 days as it had been at 48 hr. Additional evidence that the recrudescent reaction is due to host sensitization was derived by transferring I.D. homologous lymphocytes and skin simultaneously. The initial lymphocyte reaction faded, leaving a healthy skin homograft, but when the skin graft was rejected on the 10th day there was a marked inflammatory reaction at the I.D. injection site (Fig. 10). The fact that killed cells failed to produce an early reaction and yet were involved in an inflammatory reaction at 10 days again supports the different nature of the early and late reactions. The consistently smaller reactions produced by uremic cells would fit the GVH theory, as uremic cells to our knowledge are fully antigenic and should be reacted upon normally, were this an HVG reaction. Lastly, in a patient with ataxia-telangiectasia, whose immunological reactivity to homografts of skin was depressed and to dinitrochlorobenzene was absent, the injection of homologous lymphocytes produced a normal reaction. This evidence, we feel, is strongly in favor of the initial reaction's being of the GVH type and supports this conclusion reached by Brent and Medawar (1963), the results of whose experiments with guinea pigs can be interpreted in no other way.

The host's nonimmunological tissue reactivity, however, certainly plays a part in the initial reaction, so that in some individuals all reactions were larger than in others. By using a number of controls some measure of this variable can be arrived at. Evidence against this reaction's being on a GVH basis has been presented by Amos, Peacocke, and Sieker (personal communication). In one experiment, immune cells were injected into normal and into immune recipients and normal cells were injected into normal and into immune recipients, with the contention that if it were a GVH reaction the greatest reactions would be produced by immune cells and if there were a strong HVG element the greatest reactions would be seen in the immune recipients. In fact, the largest reactions were experienced by normal recipients and the smallest were consistently given by immune cells in immune recipients. The latter result might be explained by the particularly rapid destruction of the injected cells by the hyperreactive host and the failure of the immune cells to produce the largest reactions may be due to the nonspecificity of the transfers.

A more telling objection to the GVH hypothesis can be found in the result of another experiment by Amos *et al.* (personal communication), who found that PMN leukocyte suspensions containing 15 to 20 percent lymphocytes produced results which agreed closely with those obtained with a similar total injection dose of almost pure lymphocytes. We have found, however, that PMN suspensions may produce nonimmunological reactions in autologous recipients and presumably also in the homologous situation, which could go some way toward explaining Amos's results, though one must appeal to chance to explain the close agreement he had found.

The transfer of lymphocytes sensitizes the host. Evidence of sensitization was seen at 6 to 8 days, was marked at 14 days, and had disappeared by 5 months. This sensitivity lasts longer than that produced by the leukocyte extracts used by Lawrence *et al.* (1960), and is presumably explained by a difference in antigen dose. Although this sensitization makes possible an accelerated reaction against the host by any immunologically competent cells carried by an organ later grafted from cell recipient to donor, this theoretical objection carries little danger, at least in kidney transplantation (Porter and Calne, 1960).

It is worth emphasizing the importance of having a highly purified lymphocyte preparation. Both PMN and platelet suspensions often produce reactions which we believe to be pharmacological in nature, even in autologous recipients. It may be true, as stated by Bridges, Nelson, and McGeown (1964), that there is no close relation between the reaction obtained and the number of lymphocytes injected or with the proportion of contaminating granulocytes, but the latter factor clearly adds a potential nonimmuno-

logical variable which tends to reduce the specificity of the reactions.

Judged by the results of the skin-graft transfers, the discriminatory power is not high. In nine out of 17 pairs the predicted order was correct, in five cases the grafts were rejected on the same day, and in two the order was reversed. In two of the three wrong predictions we believe a technical error was the cause, for the lymphocyte transfer failed to sensitize the host. The majority of the reactions fall in a relatively narrow band from + to ++. Using skin as the test organ, a + difference has not proved a reliable guide to the order of rejection. In the few instances when we have had the opportunity to compare greater than + differences, the prediction has been correct. Though there is wide scatter of rejection times in human skin grafting, 7 to 17 days in our hands, compared with 6 to 19 in Lawrence's (Lawrence *et al.*, 1960), the majority of grafts are rejected in 10 to 12 days, which does not offer a sufficient range to assess the discriminatory powers of the test. In fact, the lymphocyte-transfer reactions may in some instances be revealing histocompatibility differences which cannot be detected by the use of skin-graft rejection times in the unmodified host. It may be that if it had been possible to increase the scatter of survival time by immunosuppressive agents there might have been a better correlation with the reactions. An insufficient number of kidney donors has been selected on the basis of the test to allow a reliable conclusion concerning its efficacy for this purpose. Although we agree with Bridges, Nelson, and McGeown (1964) that uremic cells produce generally less marked reaction than normal cells, making their use in a discriminatory test more difficult, nevertheless, the reactions produced by these cells have correlated well with the known genetic relationship of donor and recipient.

In conclusion, we believe that this test is soundly based theoretically, but that its practical value is limited by the narrow range over which the majority of reactions are spread. It can eliminate donors who have particularly strong histocompatibility differences with the recipient but is probably not sufficiently sensitive to select reliably the best donor from a panel of blood relatives. In the absence of definitive methods of full tissue typing we feel justified in continuing to use the NLT test and in accumulating clinical cases whose donors have been selected on this basis (other factors concurring), in order to further evaluate it.

Summary

Viable lymphocyte suspensions of greater than 90-percent purity have been prepared from human venous blood. The reaction produced by the intradermal injection of homologous lymphocytes has been studied both macroscopically and microscopically.

An attempt has been made to assess the relative importance of immunologic reactions by the graft or the host at various stages.

The specificity of the heightened reactivity produced by the injection of foreign lymphocytes has been estimated by the use of skin-graft survival times.

The usefulness of the test in selecting donors on the grounds of histocompatibility has been judged by transferring skin grafts from cell recipients to cell donors and attempting to predict their order of rejection. In a small number of cases, the test has been used to help in selecting human kidney donors. We believe that the reaction as read at 48 hr is a measure of the genetic disparity between donor and recipient and is largely GVH in nature. The sensitivity of the test as judged by reciprocal skin-graft survival times is not high, but might appear higher were a better system for assessing it available. At the present time we consider that the test has some value, at least in excluding donors unsuitable on the grounds of histocompatibility.

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Discussion

DR. CARPENTER: At the Peter Bent Brigham Hospital we have been pursuing similar tests. In fact, I don't think there is any major difference in our preparative technique for the NLT test or in our general interpretation of the results. We have set up some of our panels of normal human volunteers in a different way and it might be of value to compare these with those that have been presented.

(Table 1) We thought we would choose as a model a panel which would resemble the clinical situation in which four prospective kidney donors would be evaluated against a single recipient, following Brent's guinea pig experiments. In the three panels represented as A, B, and C in the table we chose four entirely unrelated people, realizing, of course, that there might be very little chance of finding a compatible individual at random. Each panel was an independent experiment, and the numbers refer to the members of the individual panels. We attempted to rank the NLT test in order of magnitude and by statistical means have indicated at the 5-percent confidence level to what degree we could separate these reactions as being truly distinct from one another. In panel A individuals 3 and 2 had identical reactions, greater than those of 4 and 1. So here we could actually distinguish only two sizes of reactions. Comparing the NLT results with skin-graft rejection times, we could say that 3's skin graft was predicted by the NLT test to be the most incompatible. On the other hand, 2, whose skin test was of equal size, turned out to have the longest survival with a difference in onset of rejection of 6 days. In panel B we again had two skin tests that were essentially alike, and, in looking at the correlation with skin-graft rejection, we find in one instance, that of individual 3, that we have a reverse correlation, in that the smaller skin test was the first to reject at day 6. We could also say, on the other hand, that individual 1, who should have been more compatible, had a maximal NLT test. In the third panel, however, we do seem to

TABLE 1

Normal Lymphocyte Transfer (NLT) : Panels of Volunteer Subjects and Correlation with Skin Graft Rejection*

Lymphocyte Donor	*Rank Order (Subject Number)*		*Comment*
	N.L.T. Test On Skin Graft Donor†	*Skin Graft Rejection on Lymphocyte Donor (Range) #*	
A	3 = 2 > 4 = 1 ($p < 0.05$)	3 > 4 > 1 > 2 (9–15d)	1) "Predicts" Incompatibility of Subject No. 3 2) Fails to "Predict" Compatibility of Subject No. 2
B	1 = 4 > 2 > 3 ($p < 0.05$)	3 > 4 > 2 > 1 (6–10d)	1) Fails to "Predict" Compatibility of Subject No. 1, and Incompatibility of Subject No. 3. (? Random)
C	1 = 2 = 3 > 4 ($p < 0.05$)	1 > 2 = 3 > 4 (8–10d)	1) "Predicts" Compatibility of Subject No. 4.

* Panels consisted in each instance of one lymphocyte donor A, B, C, and four lymphocyte recipients (1, 2, 3, 4), who also served as donors of full thickness skin grafts placed onto original lymphocyte donor.

† Ranked in order of degree of induration at 48 hours.

Ranked in order of onset of rejection as determined by gross inspection and histology with range of onset of rejection among grafts (in days).

segregate out one poor reactor (4) whose skin graft clearly survived longer than the others.

(Table 2) In addition, we took a group of four successful homotransplants at varying periods from 5 months to 5 years following transplantation and reacted their lymphocytes against their specific donors and in some instances against other people. We were interested in whether or not homotransplantation of the kidney does in fact sensitize, and particularly in whether those patients on immunosuppressive therapy would show depression of the ability of their lymphocytes to mount the NLT reaction. The asterisks refer to the specific donor-recipient combinations and one can see that D.M. had the greatest skin-test reaction against his father. These tests were performed several months after transplantation, at which time all of these recipients had normal renal function. Note J. R., representing a dyzygotic-twin pair doing extremely well on no therapy 5 years after transplantation. However, we had some evidence of mild rejection and this reaction of 30-7 mm was in our experience a fairly vigorous reaction. The figures (30-7) refer to erythema and induration, in millimeters of transverse diameter. One could look at this table in many ways and say for example that H.R. reacted poorly against all three people. We certainly do not have enough test results here to come to too many conclusions, except to say that successfully transplanted patients are *not* tolerant with respect to the NLT reaction against their specific donor.

DR. SHULMAN: If that's a graft-*vs.*-host reaction, why should it change?

DR. CARPENTER: We don't know if it is changed. We were not doing this test prior to transplantation of these individuals.

DR. SHULMAN: What was the implication?

DR. CARPENTER: We have rarely seen a reaction of this size in anybody. This is a very large reaction by the cells of J.R., and also of D.M. Our main point would be that lymphocytes from patients successfully tolerating a kidney with normal renal function are not tolerant of specific donor tissue.

TABLE 2

Reactivity of Lymphocytes after Sensitization by Renal Homotransplantation (48 Hour) (Erythema-Induration in Man)

Patients	*Skin Test Reactions*					
	L.R.	*M.R.*	*M.Z.*	*A.M.*	*G.C.*	*A.R.*
H.R.	•4–1	4–0	7–0			
D.M.	5–4			•12–9	12–6	
R.C.		7–5		13–7	•6–3	
J.R.						•30–7

We've also had the opportunity to study the effects of uremia on the lymphocyte by using an identical-twin pair. We took the cells from the two individuals while one was uremic and one was healthy, reacted them in the skin of two other people, and showed quite clearly that identical numbers of cells, with the same viability by the trypan-blue exclusion tests, were much less capable, when uremic, of mounting the graft-*vs.*-host response. One month following transplantation, when renal function was normal, both sets of cells were almost equal but there was still a slight depression of the cells from the uremic twin.

Table 3 illustrates some of the problems in applying this and other tests to transplantation in our patients. Twelve donor-recipient combinations who have had renal transplantation at our hospital are represented in the table and are ranked more or less in the order of severity of rejection. The individuals at the top have had very massive rejections, while those below have had little or no rejection. We have divided them into three main groups, severe, moderate, and minimal. The parentheses indicate the donor and one can see right away that this study is prejudiced in favor of blood relatives, all but two kidneys were obtained from the father or mother of the patient. One donor was a wife, and one was an unrelated hydrocephalic child. First of all, we applied the "third-man" triple-graft technique and it can be seen that there was no clear-cut pattern of prediction in terms of rejections later encountered. In fact, the distribution appears quite random. We had quite a large percentage of white grafts. (The interval between grafts was 14 days.) Reverse grafts were done in a few cases from the recipient back to the donor. The only notable thing here was that two reverse grafts were prolonged, one from a son to his father and the other from a daughter to her mother. In the fourth column is the NLT test at 48 hr, again in millimeters of erythema and of induration. With G.J. we had quite a large response, while W.K. did not produce a very large reaction against his prospective donor.

As we look down the table we find that there are scatterings of very low to negative NLT results. C.S. is one patient in the group doing very well after a rather large reaction. We wondered if this might represent presensitization due to blood transfusions. Although many kidney donors also received skin grafts as well as lymphocytes from the prospective kidney recipient, these tests were done on the same day, so that there should have been no presensitization of the skin-test recipients. We don't feel that we have enough data, however, to explain away every case in which the NLT test failed to predict the outcome of renal transplantation. We feel that perhaps the uremic factor is the main one which has given us such great difficulty in using this as a screening test. Although we do like to see negative or minimal reactions, we cannot be certain of the role of uremia in such cases.

DR. AMOS: In an attempt to decide if the reaction was primarily graft-*vs.*-host, we have worked with cells from normal subjects and from subjects immunized in the way I mentioned this morning. There are certainly variations between subjects receiving either immune or normal cells, but there was no consistency on attempts to repeat a given donor-recipient test and little evidence of an enhanced reaction after immunization. Nonspecific factors such as release of and sensitivity to permeability factors could obscure any significant differences as this test is at present carried out.

DR. GRAY: I would just say that it is awfully difficult finally to assess a test in a

TABLE 3

HISTOCOMPATIBILITY TESTING

Correlation of Pre-operative Skin Grafting and Normal Lymphocyte Transfer with Renal Homograft Rejection. Follow-up 2-6 Months

Degree of Rejection	*Recipient (Donor)*	*Triple Graft*	*Reverse Graft Onset of Rejection*	*48 Hr. N.L.T. Test Erythema-Induration*		*Comments*
Severe	G.J. (Wife)	Partial White	10 days	14mm.	8mm.	Rejection Reversed with Prednisone 400mg./da.
	W.K. (Mother)	White	8	4	4	Rejection Reversed with Prednisone 400mg./da.
Moderate	D.P. (Mother)	White	—	5	4	
	B.S. (Mother)	Partial White	—	7	2	
	M.B. (Mother)	Accel.	28+	0	0	
	K.R. (Mother)	Accel.	12	3	0	
	L.B. (Father)	White	—	—	—	
Minimal	S.W. (Mother)	White	7	6	3	Complicated by Recurrent Glomerulonephritis
	C.S. (Mother)	Partial White	—	12	7	Rejection at 3 Days Easily Reversed (Possible Pre-Sensitization)
	E.T. (Mother)	Partial White	—	6	0	
	A.H. (Father)	White	22+	7	3	No Clear Cut Rejection
	M.B. (Unrelated)	Partial White	—	0	0	No Clear Cut Rejection

retrospective manner where there is a possibility of presensitization by the kidney or by a prior third-man skin-graft transfer.

DR. BRENT: On the question of whether the NLT test is a graft-*vs.*-host reaction, Medawar and I have done the sort of experiments which unfortunately one can't do on human beings. Using inbred strains of guinea pigs, we injected parental-strain lymphocytes into F 1 hybrids and, conversely, F 1 hybrid lymphocytes into animals of either of the two parental strains. As expected, we found that parental-strain cells in F 1 animals gave a perfectly good NLT reaction, whereas F 1 hybrid cells failed to react in recipients of the parental strains. This, I think, shows conclusively, at least in the guinea pig, that we are dealing with a graft-*vs.*-host reaction. Dr. Gray's and Dr. Russell's evidence in human beings is, to my mind, also pretty convincing.

As far as the problem of uremia is concerned, I wonder whether Dr. Gray, or anyone else here, has tried to pep up uremic

cells in any way, perhaps simply by a few hours of *in vitro* incubation before transfer; such a procedure might conceivably elute from the cells some toxic or inhibiting substances present in the blood of uremic individuals.

DR. HIRSCHHORN: This isn't exactly an answer to your question, but after prolonged incubation, during exposure to stimulating agents such as phytohemagglutinin, the response is not inhibited in uremic cells. So the idea of washing out would seem to be good.

DR. LOWENSTEIN: I was going to mention it in this next paper. In the mixed leukocyte reaction where we have used a uremic patient's plasma with normal leukocytes we found there was very poor growth. When we substituted normal plasma, this reaction was much improved.

DR. RUSSELL: I have nothing to add, although I think Dr. Brent's suggestion that this might be done is a good one. Of course, the amount of rehabilitation that one might get would be unpredictable but would be taken care of by the controls of the test if one is just looking for a comparative response. Any degree of rehabilitation would be a good thing because it may develop more striking differences, which we are seeking. Therefore, I think it is quite a reasonable thing to do.

DR. MERRILL: I would like to make one practical comment which I think may be apropos of tissue typing as we attempt (at least I do) to relate it to transplanted kidneys. That is, that probably the way to prepare the uremic lymphocytes for the lymphocyte transfer test is to prepare the whole patient. This way you not only prepare his lymphocytes for the test but you prepare the patient for surgery, and this is perfectly practical with the present methods of dialysis. Having had some experience in studying metabolism of uremic cells *in vitro,* I think that any *in vitro* attempt to modify them might well impair the value of such a test. We suggest, if you are thinking of doing it, that you prepare the patient as a whole rather than the individual's cells.

DR. BRENT: It might be of interest that Patel and Moorehead in Liverpool have been using the NLT test on a large number of individuals in order to see whether there is any correlation between the presence of the major blood groups in the donor or the recipient and the strength of the NLT reaction. They have found no correlation. Nor does the sex of donor and recipient influence the outcome of the test.

The *In Vitro* "Mixed Leukocyte Reaction" and Initial Studies in its Application as a Test for Histocompatibility*

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Introduction

In previous publications (Bain, Vas, and Lowenstein, 1963, 1964), we have shown that, when mixtures of leukocytes from two unrelated subjects are cultured for several days, large immature cells, probably derived from small lymphocytes, appear in the cultures. These cells synthesize DNA and undergo mitosis. This occurs in the absence of any external stimulating agent, such as phytohemagglutinin.

From H^3-thymidine autoradiographs, we have found that only the large immature cells synthesize DNA; consequently, their presence can be detected by measuring H^3-thymidine uptake in the cultures.

These large cells are very similar to the basophilic and pyroninophilic cells found in lymph nodes and spleen during immune response to soluble antigens (Leduc, Coons, and Connolly, 1955) or to skin grafting (Andre *et al.*, 1962). Also, they resemble cells found *in vitro* in leukocyte cultures from single individuals after the addition of certain antigens (Pearmain, Lycette, and Fitzgerald, 1963; Elves, Roath, and Israels, 1963; Lycette and Pearmain, 1963). Because of these similarities, we suggested that this reaction in leukocyte mixtures may be a response to the presence of foreign histocompatibility antigens, which could be used as a means of tissue typing (Bain, Vas, and Lowenstein, 1964). Subsequently, Dr. Kurt Hirschhorn and his co-workers published data which led them to conclude that the mixed leukocyte reaction could be used as a method for estimating tissue compatibility (Bach and Hirschhorn, 1964; Rubin *et al.*, 1964).

This report concerns two aspects of our work with leukocyte mixtures:

(1) A series of experiments with sibling pairs. The reaction in tissue culture between their leukocytes has been quantitated, and compared with the reaction between the leukocytes from each member of the sibling pairs and an unrelated subject.

(2) Some preliminary experiments with hospital patients and normal subjects who have exchanged small skin grafts. These studies were done with two aims: first, to determine whether the amount of reaction between two subjects could be correlated with the survival times of their grafts; and, second, to determine whether the mixed leukocyte reaction was enhanced after grafting and could be related to the time of homograft rejection.

Methods

Leukocytes were separated from heparinized venous blood by allowing the red cells to sediment for 30 to 60 min. The tubes were centrifuged at 25 g (300 rpm) for 5 min. The supernatant plasma containing the leukocytes was drawn off, and the white cells were counted. The supernatant was diluted with 199 medium to produce a leukocyte count of approximately 1000/mm³. The final plasma dilution was 4 to 10 times, cell-free plasma being added if necessary. All cultures had a volume of 4 ml; the mixtures contained equal volumes of cell suspension from each subject. The culture tubes were incubated upright at 37°C and by the end of 24 hrs all the cells settled to the bottom. In every experiment, unmixed control cultures also were prepared with leukocytes from each subject alone.

In some of the grafting experiments, the lymphocytes were concentrated and cultured. Monocytes and neutrophils were removed by allowing the cells to settle onto the side of a flint-glass bottle. When the bottle was stood upright again, the proportion of lym-

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** J. B. Collip Fellow, McGill University.

phocytes in the fluid had increased, because most of the monocytes and neutrophils remained attached to the wall of the glass bottle.

After 5 days' incubation, H^3-thymidine was added to the cultures to give a concentration of 1 μc/ml. One hour later, the cells were washed three times with cold saline, then digested with Hyamine for 2 days, and the radioactivity of the resulting clear solution was measured with a liquid scintillation counter.

In the experiments with sibling pairs, H^3-thymidine uptake was also estimated by counting the proportion of labeled cells in autoradiographs. The thymidine was added 1 hr before the smears were made. The slides were fixed in absolute methanol and coated with Kodak NTB2 liquid nuclear-track emulsion. They were exposed for 2 weeks, developed, and stained with Giemsa stain.

Results

Figure 1 demonstrates the striking increase in H^3-thymidine uptake that occurs in mixtures, as compared with unmixed controls. These results are from autoradiographs. Liquid scintillation counting shows a similar picture.

1. Sibling Pairs

Fifteen normal sibling pairs were studied. Their leukocytes were mixed together, and leukocytes from each member of the pair were mixed with those from a third, unrelated subject. The reaction in the mixtures was measured both by autoradiographs and by liquid scintillation counting.

Figure 2 shows the autoradiograph results. For each point, the ordinate represents the reaction between an individual and his sibling, and the abscissa represents the reaction between the same individual and an unrelated subject. Each sibling pair is represented by 2 points on the graph. The points would lie on the diagonal line if sibling pairs and unrelated pairs reacted equally. Points above the line indicate a stronger reaction between siblings, and those below the line indicate a stronger reaction between the member of a sibling pair and an unrelated subject. The small point with a circle represents the mean of the sibling pairs' values and the mean of the unrelated pairs. Only five of the 30 points are above the diagonal line. A t-test showed that the mean difference between sibling-unrelated subject mixtures and the corresponding sibling pair mixtures was significantly greater than zero, p being less than 0.01. Similarity or difference in sex did not appear to affect the results.

One point is on the diagonal line, and the other 24 are below it. Ten of the points are open circles. These represent five sibling pairs whose leukocytes showed no reaction when compared with the unmixed controls. Some of the nonreacting pairs were

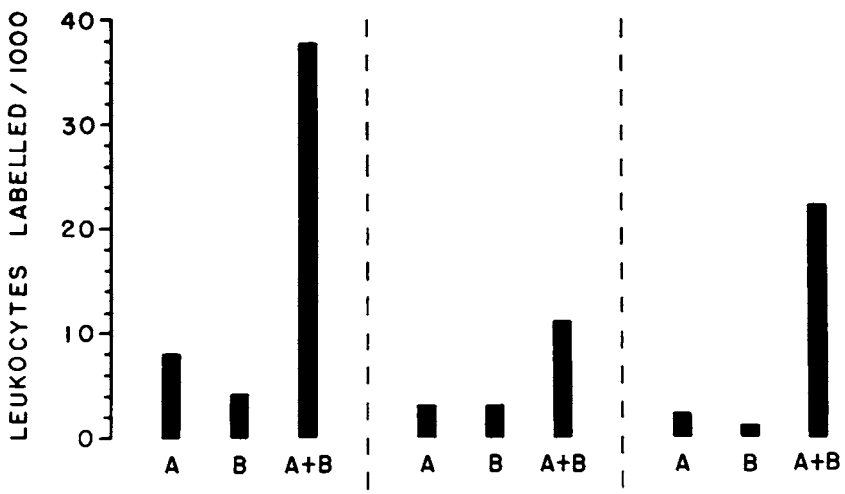

Figure 1.—H^3-thymidine autoradiographs of 5-day leukocyte cultures from three unrelated subjects. In each experiment, A and B are the unmixed controls; A + B is the corresponding mixture. (From Bain, Vas, and Lowenstein, 1964.)

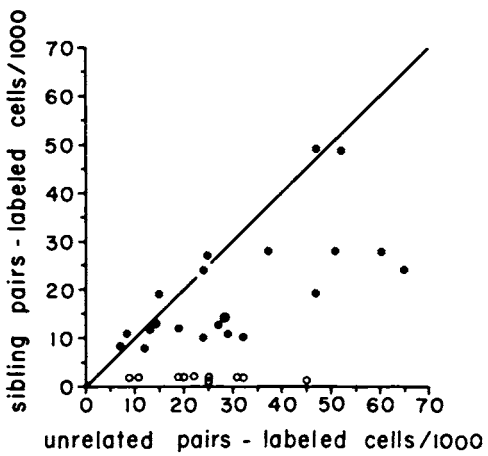

Figure 2.—H^3-thymidine autoradiographs of leukocyte cultures from 15 sibling pairs, and from each member of these pairs and an unrelated subject. Ordinate represents labeled cell counts of sibling pairs; abscissa represents the labeled cell counts of each member of a sibling pair and an unrelated subject. Small point with circle represents the mean of these values. If sibling pairs and unrelated pairs reacted equally, their points would fall on the diagonal line. Open circles represent five sibling pairs whose leukocytes showed no reaction compared with the unmixed controls.

studied a second time, and again showed no reaction. Similarly, other sibling pairs consistently showed a positive reaction. More than 100 pairs of unrelated normal subjects have been studied so far, and none have failed to react.

The results with liquid scintillation counting are depicted in Fig. 3. The symbols are the same as in Fig. 2, and, as before, there are only five points above the diagonal line. The values show a greater range than the autoradiographs, but the difference between sibling pairs and unrelated pairs is statistically significant.

The experiments with siblings were performed over several months. During this time, a number of technical modifications may have contributed to the variability of the results. For example, H^3-thymidine solutions of different specific activities were used, a change was made in the buffering system of the medium, and a new liquid scintillation counter was installed.

There was some overlap in the liquid scintillation counts of the reacting and nonreacting sibling pair mixtures. They were differentiated by a comparison of the counts in the mixtures with the counts in the unmixed controls. Furthermore, all sibling pairs classified as "nonreacting" also showed no evidence of a reaction either from autoradiographs or from the examination of stained smears.

It is concluded from these studies that the mixed leukocyte reaction tends to be diminished when the two subjects are siblings. Some subjects, however, react as strongly with their siblings as they do with unrelated individuals. Other sibling pairs show no reaction that can be detected by the methods employed in these experiments.

From the laws of inheritance, it would seem possible that this test might be used in homotransplantation to select a highly compatible donor from among the recipient's brothers and sisters. The degree of histocompatibility between parents and children may be limited by the fact that a child always inherits half of its chromosomes from each parent. On the other hand, siblings may be genetically closer or farther apart than this. The sibling pairs whose leukocytes do not react in mixtures may be extremely similar to one another and might exchange tissues with relatively good chances of success.

This hypothesis should be tested by comparing the results of the mixed leukocyte reaction with the results of skin grafting between pairs of siblings.

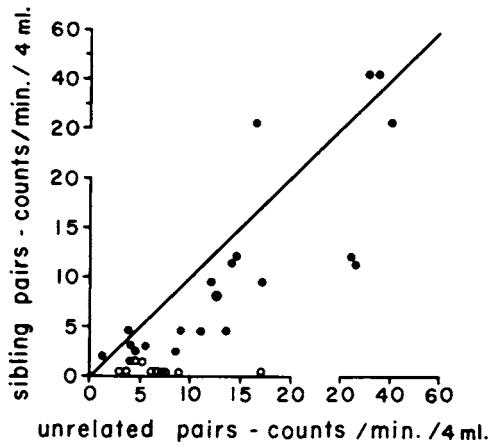

Figure 3.—H^3-thymidine liquid scintillation counts of leukocyte cultures from 15 sibling pairs, and from each member of these pairs and an unrelated subject. (See Fig. 2.)

2. Skin-Grafting Experiments

These experiments were planned with two objectives in view:

(1) to determine whether the degree of reaction between the mixed leukocytes from a particular pair of subjects could be correlated with the survival time of their crossed skin grafts; and

(2) to determine whether the mixed leukocyte reaction is enhanced after grafting, and, if so, at what time the enhancement occurs in relation to the time of rejection.

It was hoped that the information obtained would indicate the feasibility of using the mixed leukocyte reaction to predict homograft compatibility, its value as a means for detecting impending rejection, and its possible use as a guide to dosage of immunosuppressive therapy.

The subjects used in the experiments were five pairs of normals (nine males, one female) and three pairs of hospital patients (five males, one female). The latter had a variety of conditions:

Pair 1: (a) open heart surgery, postoperative
(b) carotid-artery thrombosis, recovering

Pair 2: (a) diabetes
(b) leg amputation, osteomyelitis, draining stump

Pair 3: (a) carcinoma of colon, postoperative
(b) carcinoma of colon, postoperative

The pairs of subjects exchanged full-thickness skin grafts, 1 cm in diameter. The day was noted on which the first signs of rejections were visible by eye. The time of complete rejection was not noted as accurately. Blood was taken from each pair, three times before grafting, and once or twice weekly after grafting for up to 8 weeks. Mixed cultures and unmixed control cultures were prepared. The mixed leukocyte reaction was measured by liquid scintillation counting, and the results were expressed in two ways:

(1) counts/min/mixed culture, and

(2) In an attempt to cancel out some of the technical variability, the count on the mixture was divided by the average of the two unmixed control counts.

a. *Comparison of the Mixed Leukocyte Reaction with Rejection Time.* The three pairs of patients showed an abnormally low reaction before grafting for unrelated pairs. Some, but not all, of these results could be explained on the basis of severe lymphocytopenia. The initial signs of rejection of the grafts were delayed in the patients when their survival times were compared with those of graft survival in the normals (Table 1). To this limited extent, graft-survival time was related to the intensity of the mixed leukocyte reaction.

Figure 4 shows pregrafting results on three pairs of normals whose leukocytes were cultured both with and without separation of lymphocytes. Within the normal group, there was considerable variation in the mixed leukocyte reaction before grafting, but little variation in graft-survival time. Deliberate adjustment of the lymphocyte concentration to a constant value did not make the reaction more consistent, and the counts per minute were not proportional to the number of lymphocytes originally present in the mixed cultures.

Some of the differences in reaction were undoubtedly due to technical factors, but two

TABLE 1

Relation of Graft-Survival Time to the Intensity of the Mixed Leukocyte Reaction

	Survival time, days to beginning of rejection		*Pregrafting counts/min/culture*		*Ratio of mixtures to controls*	
Subjects	*Mean*	*Range*	*Mean*	*Range*	*Mean*	*Range*
Normals (5 pairs)	7.5	5–8	23,910	5,369–64,401	15.0	4.2–28.7
Patients (3 pairs)	12	9–13+	583	86–1,104	2.5	0.7–5.1

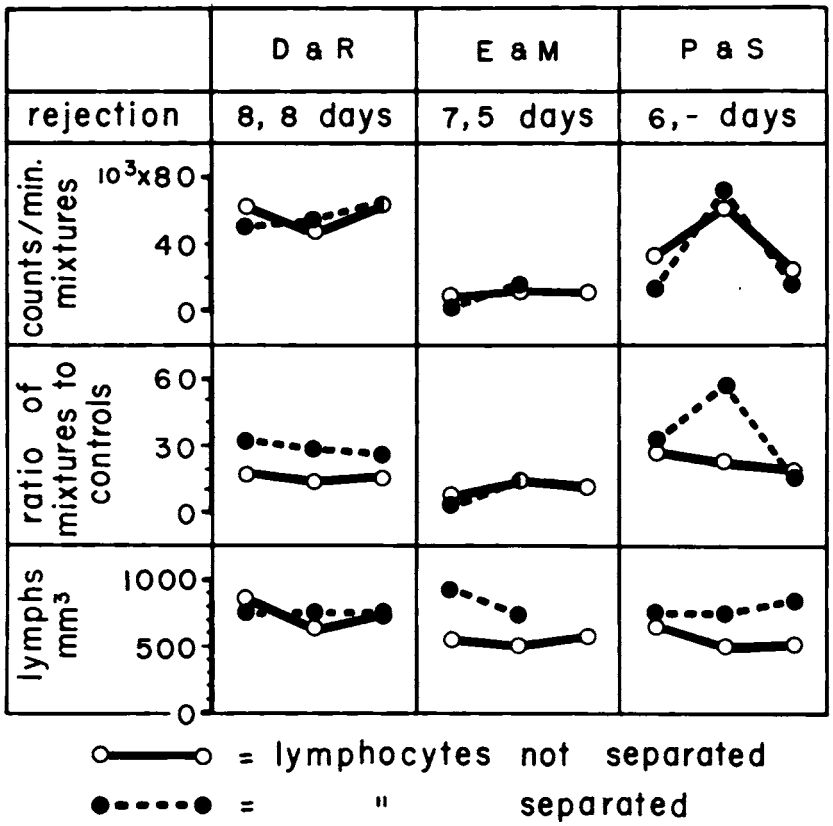

Figure 4.—Mixed leukocyte cultures. Liquid scintillation counting of intact leukocyte mixtures and of cultures of mixed lymphocyte concentrates from three pairs of normal subjects before grafting.

pairs, E&M and P&S, were studied concurrently and their results were subject to the same technical variables. The reaction in the E&M mixtures was consistently weaker than that in the P&S mixtures, both in the pregrafting period and during the initial period after grafting. E and M began to reject at 7 and 5 days, respectively, and P's graft displayed initial signs of rejection at 6 days. S's graft remained greyish and probably never became vascularized.

b. *Changes in the Mixed Leukocyte Reaction after Grafting.* The averaged results from the experiments with normal subjects are shown in Fig. 5. In view of the extreme variability of the results from each pair, most of the fluctuations cannot be regarded as significant. However, the increased reaction that occurred at 19 to 21 days after grafting was observed in four of the five pairs. In the only pair in which it did not occur (P&S), one of the grafts was considered not to have been accepted. Although the increased reaction following grafting varied in its duration, it always began after rejection was complete.

In contrast with the normals, the reaction in the three pairs of patients was decreased as compared with the normals in the pregrafting period, and increased after grafting during the period leading up to rejection (Fig. 6). Only one of these pairs (A&H) was studied for a longer time than is shown in the figure. Their maximum reaction occurred on the day that rejection was first noted, but the values had still not returned to the pregrafting levels when the last blood sample was taken at 33 days.

Discussion

The three pairs of patients whose mixed leukocyte reaction was very diminished before grafting also had a delayed onset of rejection when compared with normal subjects. Within the normal group, no correla-

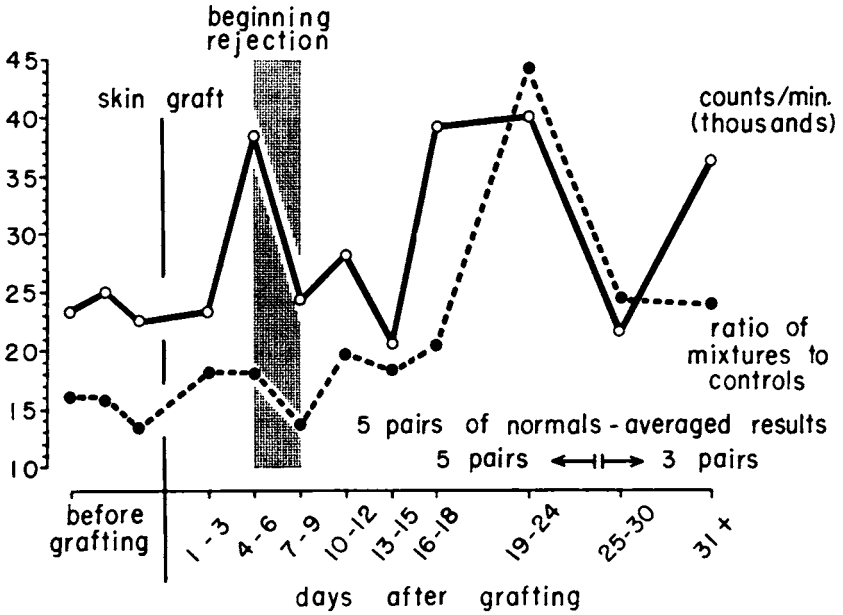

Figure 5.—The mixed leukocyte reaction and its relation to cross-skin-graft acceptance and rejection in five pairs of normal subjects.

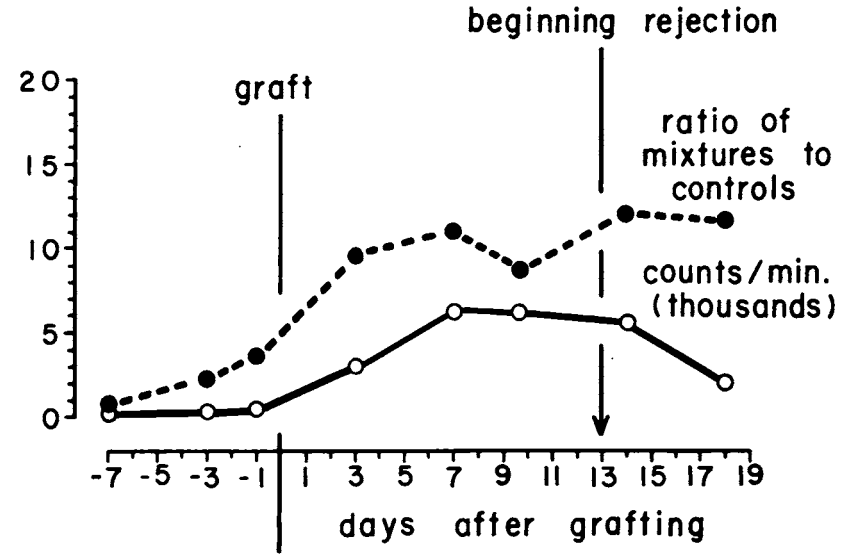

Figure 6.—The mixed leukocyte reaction and its relation to cross-skin-graft acceptance and rejection in patient pair 1.

tion could be found between graft survival and the intensity of the reaction in leukocyte mixtures. It is possible that homograft survival is relatively constant when the pregraft mixed leukocyte reaction of donor and recipient varies within a certain range, providing these variations of the reaction are not due to technical factors. However, variations in the reaction between different pairs might still reflect the relative ease with which rejection could be suppressed by such means as the use of immunosuppressive drugs.

The small degree of reaction between patient pairs was surprising, and leads one to suspect that nonspecific factors, such as

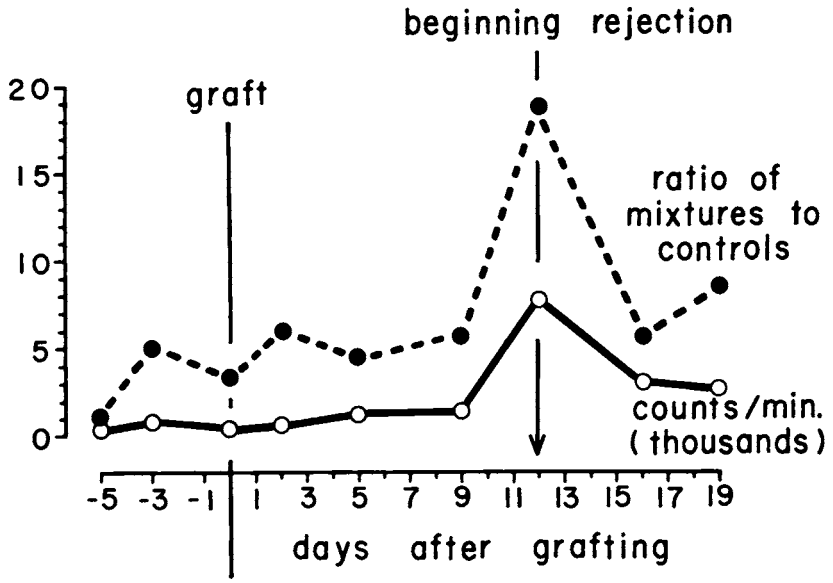

Figure 7.—The mixed leukocyte reaction and its relation to cross-skin-graft acceptance and rejection in patient pair 2.

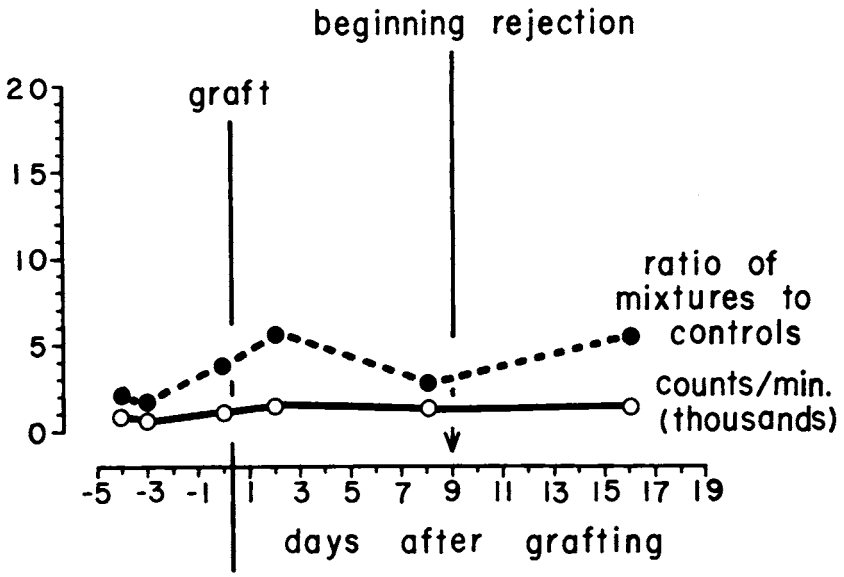

Figure 8.—The mixed leukocyte reaction and its relation to cross-skin-graft acceptance and rejection in patient pair 3.

trauma, stress, and chronic infection, may influence the results. Immune reactions are frequently depressed in cancer patients, and both members of one of our pairs had cancer of the colon.

At about 3 weeks after grafting, the leukocyte mixtures from normal pairs began to react more strongly than they had previously. Contrary to expectations, the increase never occurred until after rejection was complete. The reasons for this observation are not clear, but two possible hypotheses may be offered:

(1) during rejection, very few sensitized

lymphocytes may have been present in the circulation, because some were invading the graft and others were retained in the lymphoid tissues; or

(2) lymphocytic cytotoxic antibodies (Walford, Gallagher, and Sjaarda, 1964) may have been present and may have been responsible for suppression of the mixed leukocyte reaction until after homograft rejection was completed.

With our present techniques, it is obvious that the mixed leukocyte reaction could not be used as a means for predicting a rejection crisis in these normal subjects.

The three pairs of patients behaved quite differently from the normals. Their pregrafting leukocyte mixtures had extremely low counts, and the only indication of any reaction at all was the fact that their unmixed control counts were even lower than the mixtures. During the time leading up to rejection, the reaction increased, but seldom rose above the lower limits of the pregrafting values in the normals. The situation with the patients also is quite unclear, but one may speculate as to why their mixed leukocyte reactions increased before the onset of rejection:

(1) The increase in reaction may have been due to an improvement in the condition of the patients and, hence, would have occurred whether or not they were grafted. This is a particularly likely explanation in the case of the first pair, P&V. The pregrafting tests were done during P's 2nd postoperative week after open heart surgery, when her capacity for immune reactions may have been suppressed.

(2) The poor condition of some of these patients might have prevented the formation of the circulating cytotoxic antibodies (Walford, Gallagher, and Sjaarda, 1964) that have previously been discussed in connection with the normal pairs.

The cross-transplantation experiments were pilot studies. Their results indicate need for improvement of techniques of measuring the *in vitro* reaction and in the assessment of graft survival. Considerable progress has been made in this direction, and there were fewer technical variations in the last two pairs of normals than in the previous three pairs. The methods also need to be standardized so that leukocyte mixtures from different pairs of subjects, studied at different times, can be compared with one another.

The practical use of this test will involve hospital patients, particularly uremic patients. In these cases, further problems arise, such as severe lymphocytopenia and the possible toxic effects of uremic plasma. Experiments with lymphocyte separation in normal subjects suggest that the almost complete removal of neutrophils and monocytes may have depressed the mixed leukocyte reaction to some extent. If it is necessary to concentrate the lymphocytes when testing patients, the results should be interpreted with caution, at least until a standardized and reproducible technique can be developed. Toxic substances in the plasma could be eliminated by substituting normal plasma, or by substituting fetal calf's serum, as has been done by Dr. Hirschhorn and his associates. In one experiment, leukocytes from a man who had previously received a transplanted kidney were mixed with those from a normal subject. The mixture and the patient's unmixed control culture both contained his plasma, and both of these cultures were unsatisfactory. When normal plasma was substituted throughout, the results were much improved.

We are continuing the grafting experiments, and are incorporating several changes which may clarify some points that are obscure at present:

(1) Initial and complete rejection times are being more closely noted, and future observations will include data from color photographs and biopsies of the grafts.

(2) The leukocytes from each member of a grafted pair are mixed with those from a third, "neutral" subject, as well as with those of the other member of the pair. Also, the leukocytes of each member are cultured with phytohemagglutinin as a further control.

(3) A "neutral" plasma will be substituted for the grafted subjects' plasma, in order to eliminate any circulating cytotoxic antibodies that might arise after grafting.

(4) Blood samples will be drawn more frequently, particularly during the first 3 or 4 weeks after grafting.

(5) The pairs of subjects studied in this report, and others to follow, will be used to follow the reaction in second-set homografting.

(6) When more of the technical problems are solved, skin grafts will be exchanged between sibling pairs to determine whether compatibility between nonreacting sibling

pairs may be sufficient to allow a significant prolongation of the survival of the grafts.

Conclusions

(1) Further improvements in techniques are desirable in order to relate quantitatively the mixed leukocyte reaction to homograft acceptance and rejection.

(2) Quantitatively, the mean mixed leukocyte reaction of 15 sibling pairs was decreased as compared with the mean reaction of mixtures of leukocytes of each member of the sibling pairs with those of unrelated subjects. Five sibling pairs showed no reaction.

(3) Three pairs of patients with a variety of disease conditions showed a quantitative pregraft decrease of the reaction and prolongation of crossed-homograft rejection time as compared with five pairs of normal subjects.

(4) In the normal-subject pairs there was a consistent increase of the reaction following rejection of grafts, whereas the reaction increased in the patient pairs during the period leading up to rejection. These latter observations are preliminary, and require further explanation and extension.

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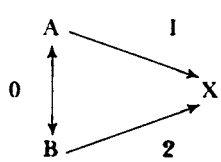
Discussion

DR. HIRSCHHORN: In the case of your five pairs of nonreactive sibs, did you find any correlation between the reactions of each member of a pair against unrelated individuals? Let us say, for example, that you have sib A and sib B who against each other show zero. You have unrelated individual X.



Is there a correlation between reactions 1 and 2?

DR. LOWENSTEIN: No, there was no definite correlation there.

DR. BRENT: Why should there be a correlation?

DR. HIRSCHHORN: Because there is no reaction between the two sibs. Do they equally recognize a foreign antigen?

DR. LOWENSTEIN: In those instances in which A and B were nonreacting siblings, B + X and A + X reactions sometimes were different and sometimes were the same.

Immune Response of Human Peripheral-Blood Lymphocytes *In Vitro**

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We have previously described an *in vitro* system for the study of the immune capabilities of human peripheral-blood lymphocytes (Hirschhorn *et al.*, 1963). The system derives from the observation that phytohemagglutinin (PHA), an extract of the kidney bean, Phaseolus vulgaris, capable of agglutinating leukocytes (Moorhead *et al.*, 1960), induces morphological alteration of small lymphocytes into large lymphocytes and plasma cells. The enlarged cells produce gamma globulin (Bach and Hirschhorn, 1963). PHA has been used to stimulate mitotic activity in lymphocytes for the purpose of chromosome analysis (Grasbeck, Nordman, and de la Chapelle, 1963). The detailed methodology is outlined on page 177 of this volume, but basically consists of separation of the lymphocytes from erythrocytes and other leukocytes, and the incubation of the lymphocytes in tissue-culture medium in the presence of PHA, specific antigens, or homologous tissue.

When human peripheral-blood lymphocytes are incubated with PHA for 72 hr, on the average, 90 to 95 percent of the cells appear as large lymphocytes, 10 to 50 percent of which morphologically and cytochemically resemble plasma cells. One to 15 percent of the cells are in mitosis. Without any additive, 5 to 10 percent of the cells are large, 0 to 0.2 percent are in mitosis, and the rest remain small. When specific antigens, to which the donor of the cells is sensitized, are added, the peak of stimulation occurs after 5 days of culture. The response, as measured by an increase of large cells plus mitoses over the percentage found in a control culture, varies from 5 to 40 percent. The degree of response is partly dependent on dose of antigen and degree of sensitivity of the donor. The antigens used have included a variety of bacterial and viral products, as well as nonprotein chemical substances, such as penicillin. The subjects tested demonstrated either delayed hypersensitivity or circulating antibodies, or both, indicating that in the isolated cell there is no apparent difference between these two types of immune response. Infants, as well as agammaglobulinemic subjects, do not respond to specific antigens, but do respond to PHA. This finding, as well as the difference in the type of response to PHA and to specific antigens, indicates that PHA is not acting as an antigen, but as a nonspecific stimulant, similar to antileukocyte antibodies (Grasbeck, Nordman, and de la Chapelle, 1963). We have found that streptolysin S, a nonantigenic product of the beta-hemolytic streptococcus, behaves similarly to PHA in all individuals, including infants and agammaglobulinemic patients. The only exceptions are patients with acute rheumatic fever, in whom the response is markedly diminished (Hirschhorn *et al.*, 1964, b). Streptolysin O, on the other hand, is an antigenic substance, and stimulates the cells of all individuals with a positive anti-streptolysin O titer, to the same extent as other specific antigens. These findings suggest that there are at least two mechanisms inducing cellular differentiation and gamma globulin production in this system. One of these involves the stimulation of specific cells with specific antigens, while the other involves generalized, nonspecific stimulation of virtually all cells, regardless of prior sensitization. Both generalized and specific stimulants also caused a sharp rise in RNA and protein (including the three types of gamma globulin production), both of which could be inhibited by appropriate doses of actinomycin D or puromycin.

* Work supported by grants from the American Heart Association (K. H., Established Investigator); USPHS (HD-00542); NYCHRC (U-1030); and Association for the Aid of Crippled Children.

While studying the question of an autoimmune component in infantile eczema (Hashem *et al.*, 1963), we found that cells from such patients showed a response of 30 to 40 percent when stimulated with extracts of their own skin or that of normal individuals. Cells from normal control subjects showed no response to their own skin, but did demonstrate a low, but variable, response to skin extracts from other, unrelated persons. This finding, as well as the report by Bain, Vas, and Lowenstein (1963), that mixed cultures of lymphocytes from two unrelated individuals caused increased thymidine uptake in the cultured cells, caused us to modify our system in order to attempt quantitative tissue typing. We were able to show (Bach and Hirschhorn, 1964) that in such lymphocyte mixtures the peak of stimulation occurs after 7 to 8 days of culture when neither donor has been sensitized to the other's tissues. Similarly, we were able to obtain stimulation by using lymphocyte extracts from either donor in cultures of the other donor's cells.

In experiments done with Dr. Rapaport's group (Hirschhorn *et al.*, 1964, a), we could show good correlation with the "third man" test for the sharing of histocompatibility genes. Individuals showing a higher degree of similarity had a lower response in lymphocyte mixtures than did individuals showing less similarity.

Mixed cultures derived from identical twins showed no stimulation: those from first-degree relatives had values of 5 to 20 percent, and those from unrelated individuals, values of 5- to 85-percent increase in large cells plus mitoses. Since we must assume that man has multiple genetically determined histocompatibility antigens, these results suggest that there is an additive effect on the lymphocytes, depending on the number of foreign antigens in the stimulating tissue.

Another factor determining the degree of response is the state of sensitization of the donor of the cells. All of our own cell-cell mixture experiments have been performed on nonimmunized individuals. However, Ceppellini, Celada, and Zanalda (in press) recently reported that if cells were derived from the donor and recipient of a previous skin graft, a response was observed at 4 days, at which time our subjects, as well as his subjects prior to immunization, showed virtually no response. This difference in the timing of the reaction led us to postulate that the interaction of cells from nonsensitized individuals represents a primary immune response *in vitro*, while the more rapid response of cells from immunized subjects represents a secondary response, similar in timing to the findings with specific antigens.

In order to test this hypothesis, we have recently performed some experiments using the technique of Ginsburg and Sachs (in preparation). They had found that spleen or thymus cells from rats had the capability of destroying mouse fibroblasts, when cultured together with these cells. The rat cells meanwhile underwent morphological changes similar to those observed in our culture system. We took human peripheral-blood lymphocytes and grew these on top of active, normal human-fibroblast cultures from unrelated individuals. PHA was added to some of the bottles (Table 1). When compared with simultaneous control fibroblast cultures with no addition of lymphocytes (Fig. 1), cultures with PHA-stimulated lymphocytes were destroyed in 3 to 4 days (Fig. 2), while unstimulated lymphocytes produced a lesser, but definite amount of destruction in 7 to 8 days (Fig 3). PHA alone, after 4 days, produced virtually no changes. When the lymphocytes were transferred to fresh fibroblasts at the time of maximum destruction, the PHA-stimulated cells, now apparently sensitized, produced destruction in 18 hr (Fig. 4), while

TABLE 1

Time of Fibroblast Destruction after Addition of Homologous Lymphocytes

Additives	*Time of fibroblast destruction*
1. Lymphocytes alone.	8 days
2. Lymphocytes from 1. transferred on day 8.	4 days
3. Lymphocytes with PHA.	4 days
4. Lymphocytes from 3. transferred on day 4.	18 hrs.

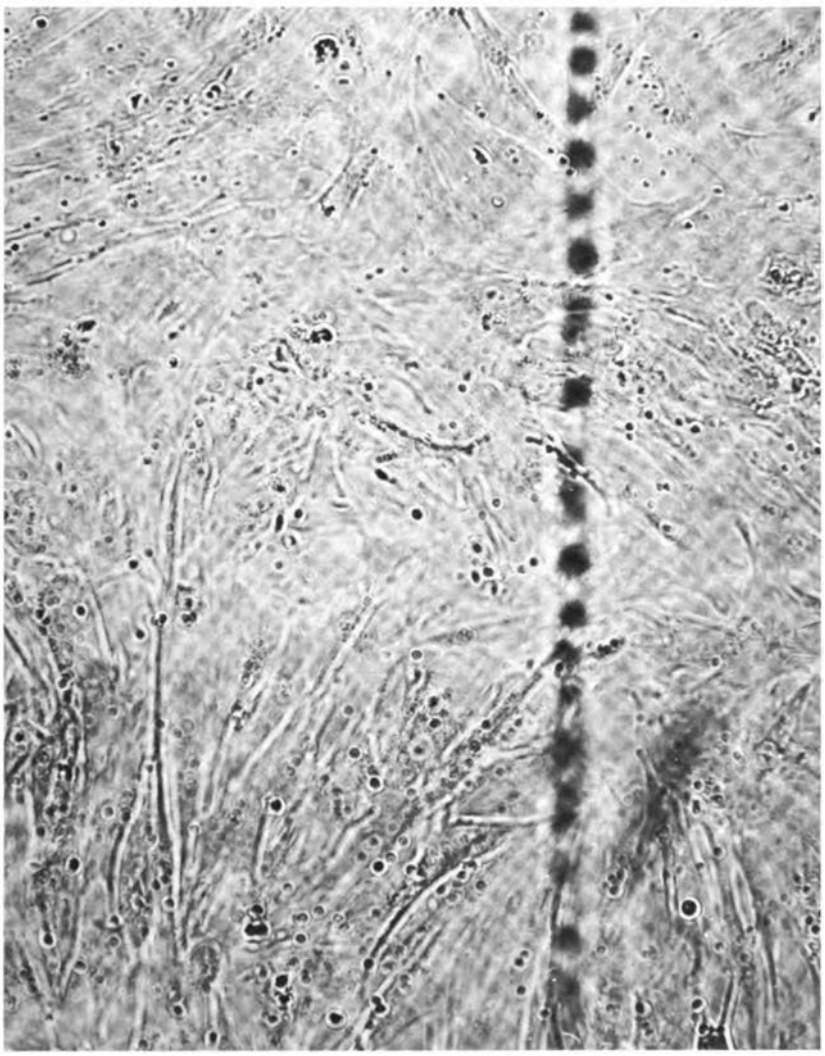

Figure 1.—Culture of fibroblasts alone. Day 3.

the transferred cells not stimulated with PHA produced this effect in 4 days (Fig. 5). These results call for two independent conclusions: (1) the time difference of response after first exposure and after transfer indicated the likelihood of primary and secondary responses *in vitro;* and (2) the presence of PHA induces a marked acceleration of both the primary and the secondary responses.

This system may have two eventual applications. First, by performing appropriate intrafamily and interfamily studies, typing may become possible, based on genetically determined differences in histocompatibility antigens.

Walford, Gallagher, and Sjaarda (1964) have recently demonstrated the feasibility of such studies by using hyperimmune sera from human recipients of multiple grafts. These sera showed variable cytotoxic effects on lymphocytes from a variety of individuals. One of the sera was used in a family study with a result indicating simple Mendelian dominant inheritance of susceptibility to cytotoxic damage by this serum. It is possi-

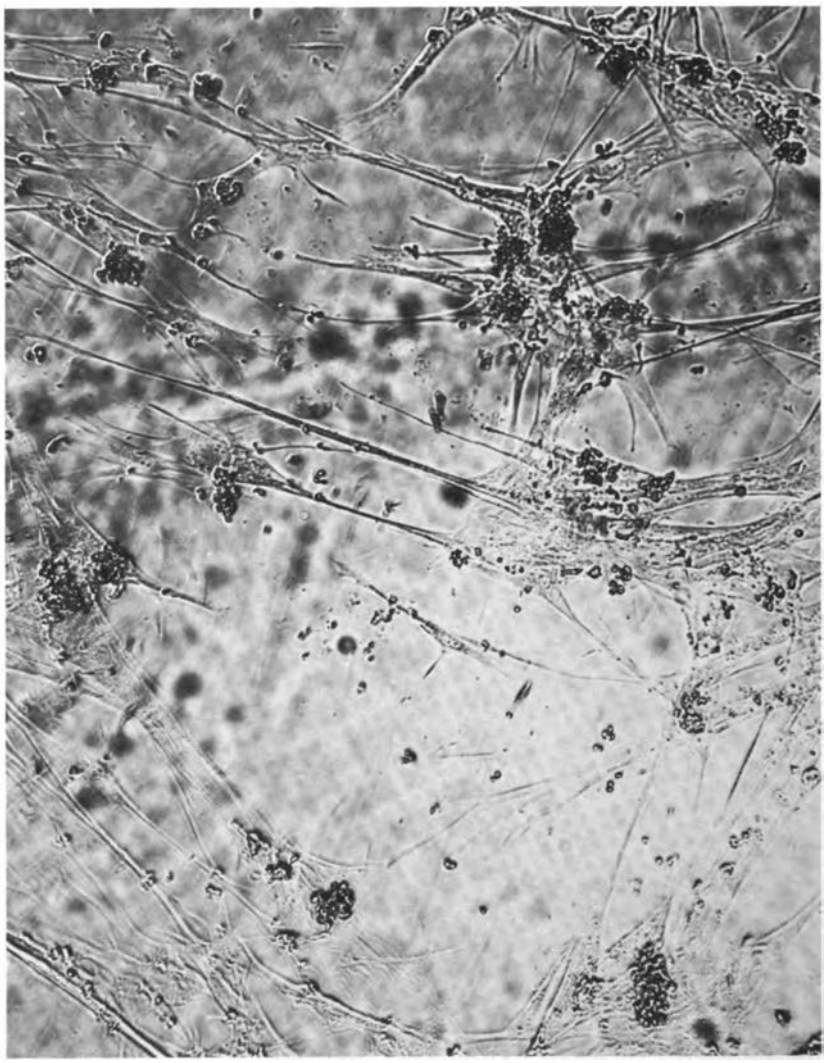

Figure 2.—Culture of fibroblasts with lymphocytes and phytohemagglutinin present. Day 3.

ble, however, to interpret Walford and coworkers' data as an example of recessive inheritance.

Secondly, and especially after a typing system is established, this method could select donors for organ transplants on a more quantitative and safer basis than is now being used. We, in the New York area, have been testing a number of donor-recipient pairs before renal homotransplantation. Of interest are two cases recently studied at Memorial Hospital with Dr. Daniel G. Miller. Both were mother-to-son grafts. One pair showed a 5-percent increase of large cells plus mitoses, while the other showed a 20-percent increase, a high response for first-degree relatives. Both recipients died 2 weeks after transplantation due to intercurrent infections. Post-mortem examination showed that the transplanted kidney of the recipient with the low response was perfectly normal, while that of the other patient demonstrated the pathology of rejection, including mononuclear-cell infiltration and necrosis.

We have observed that the lymphocytes of patients on immunosuppressive therapy do

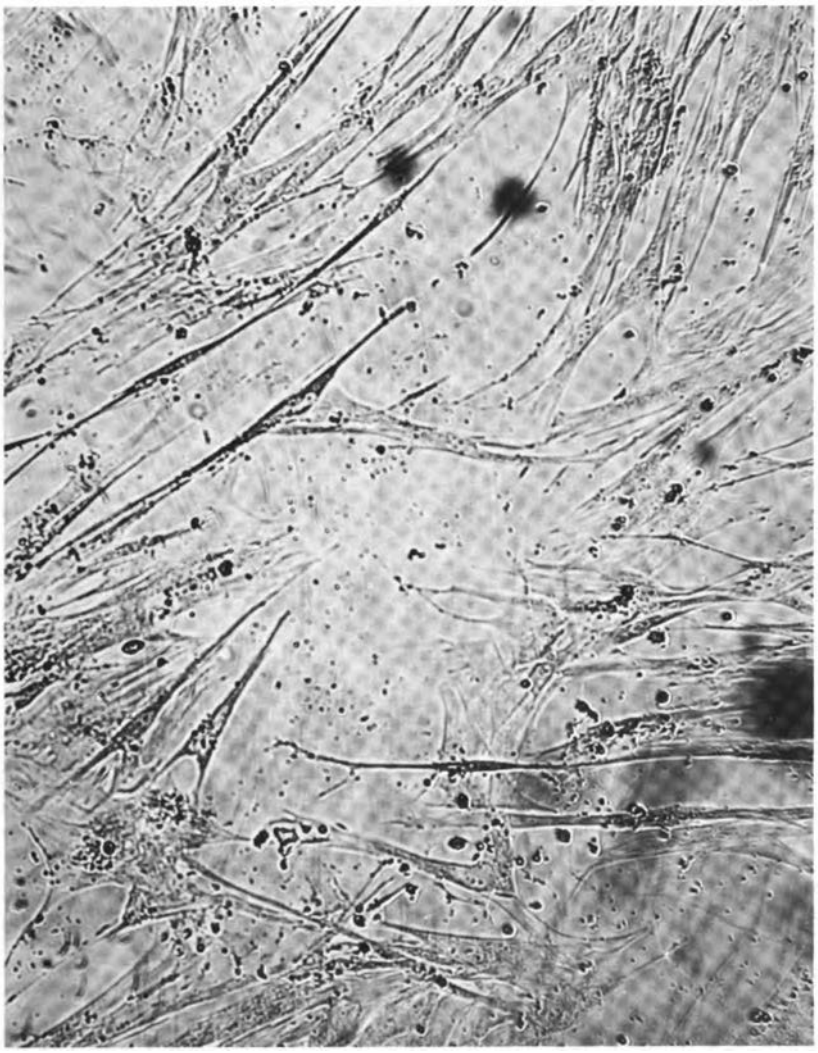

Figure 3.—Culture of fibroblasts with lymphocytes present. Day 7.

not respond to PHA (Rubin *et al.*, 1964). If, in following a patient, a response to PHA is observed, this is an indication of the onset of rejection of the graft. The return of response to PHA appears to precede clinical signs of the rejection by at least 5 or 6 days, and may be a useful screening procedure to predict the need for higher doses of immunosuppressive therapy. In studying the effects of immunosuppressive drugs *in vitro*, we had observed that actinomycin D and puromycin inhibited RNA and protein production of the cultured cells, as well as, in adequate doses, markedly reduced the number of cells in the culture. One drug which we studied was chloroquin, an antimalarial drug which has been used to suppress autoimmune phenomena in disseminated lupus erythematosus and rheumatoid arthritis. This drug, in very low doses, appears to inhibit a number of the cells from enlarging when stimulated with PHA (Hirschhorn and Hurvitz, in preparation). Since this drug is relatively innocuous *in vivo* and does not appear to affect the bone marrow as do ordinary immunosuppressive agents, it may be worth while to attempt a trial of this drug in patients receiving kidney transplants.

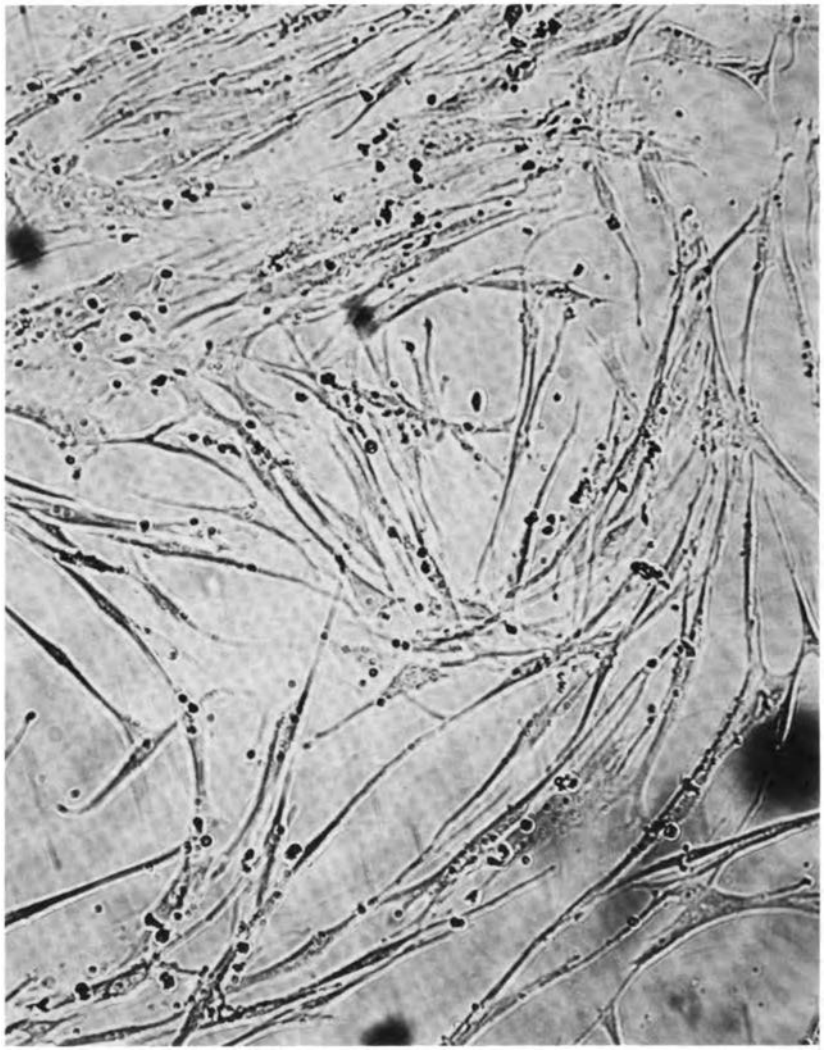

Figure 4.—Culture of fibroblasts with lymphocytes present which have been transferred on day 5 from a culture where these lymphocytes and phytohemagglutinin had been present. 18 hrs. after transfer.

The drug could be tried either alone or in conjunction with lower than usual doses of the usual immunosuppressive agents.

In summary, we have demonstrated the immune capabilities of human peripheral-blood lymphocytes and established a system of *in vitro* quantitation of their response. We have used this system to study responses to nonspecific stimulants, specific antigens, and foreign tissues. The response to foreign cells appears to represent a primary response in culture and correlates with genetic differences between individuals. It is hoped that this system may be useful in tissue typing and in the selection of donors for organ grafts.

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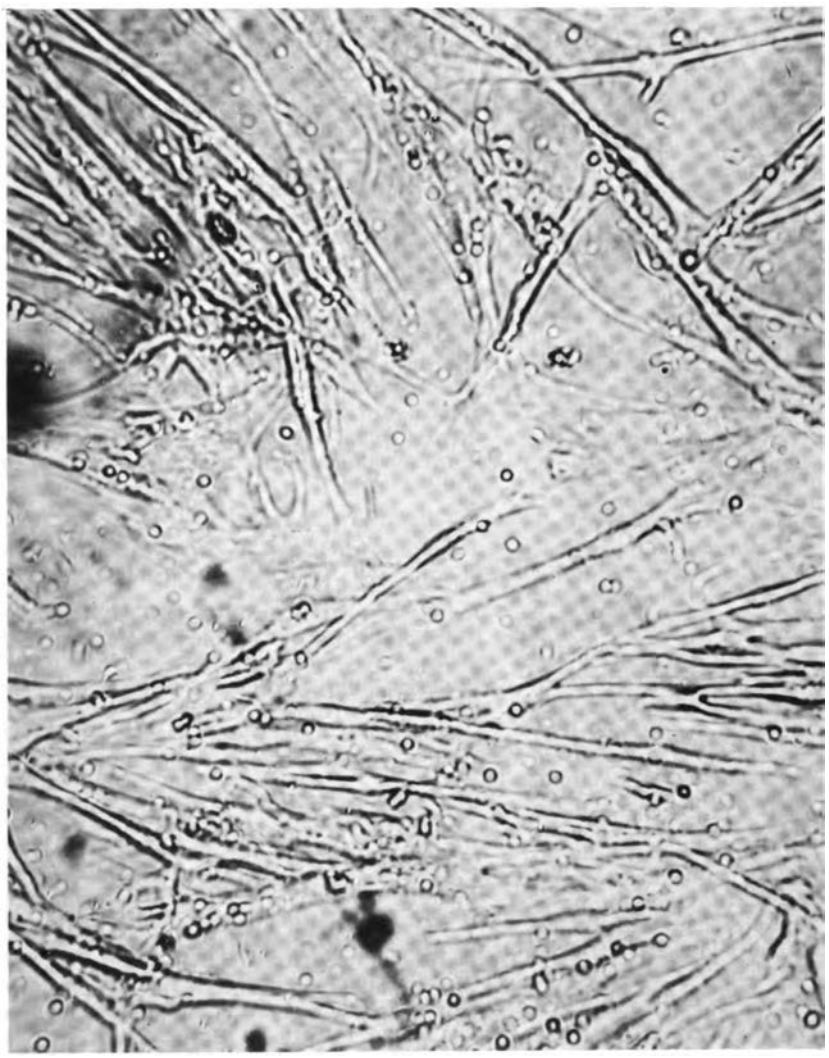

Figure 5.—Cultures of fibroblasts with lymphocytes present which have been transferred on day 8 from a culture where these lymphocytes had been present. 4 days after transfer.

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Discussion

DR. CEPPELLINI: The phenomenon of the "mixed leukocyte reaction" described by Dr. Bain's and Dr. Hirschhorn's groups is obviously of the greatest theoretical and practical importance. We too have used the test, but with varying success.

On the assumption that the reaction has an immunological basis, we were particularly interested to see if immunization of one member of the pair with white cells of the other (3×10^8 cells, intradermally) would increase the degree of activation of the mixed culture.

In a first set of experiments with normal subjects, the one quoted today by Dr. Hirschhorn but still unpublished, the reactivity (read at the 4th day) of the cultures from lymphocytes obtained 10 days after immunization was higher than for the tests run before immunization. The difficulty is that the preimmunization tests showed practically no activation of the mixed cultures. Between the preimmunization and the post immunization tests, almost 1 month had elapsed and we had in the meantime changed some of the technical details (volume of the vessel, medium, etc.), which can greatly influence the sensitivity of the test.

In a second set of experiments with cancer patients in poor general condition, the preimmunization cultures showed a good response (about 20 to 50 mitoses per 1000 cells; our technique had evidently improved). This time we harvested the cultures at the 7th day.

It is therefore impossible to draw any conclusion from our experiments about whether the cell-to-cell reaction can be bolstered by a specific stimulus. I may add that the dose of white cells injected intradermally into the recipient induces a strong homotransplantation immunity (already developed on the 10th day), as shown by the white graft or accelerated type of rejection of a subsequent skin graft from the same donor. It is also possible that at the 10th postimmunization day cytotoxic antibodies have already developed, which may interfere with the reaction.

We have also run some mixed cultures between sibs and we can confirm the results of Drs. Bain and Lowenstein, about a lower average of activation. In our hands, however, variability on repetition of the test is still very high.

Finally, we were not able to obtain any activation when one of the two populations of white cells was killed by repeated freezing.

In regard to the nature of the activation of lymphocyte cultures by phytohemagglutinin, we have been able to confirm the finding of Dr. Hirschhorn's group: 10 to 30 percent of the *de novo* synthesized proteins are gamma globulins (7S, quantitatively estimated by means of specific precipitating antisera).

DR. HIRSCHHORN: First, the failure of response to extracts or homogenates may be due to the method of preparation of the extracts. This is a matter of laboratory differences which can't be settled here, but we and several other groups have gotten very nice responses with extracts.

The second point is Dr. Ceppellini's failure to find an increase after immunization. He now apparently does all the cultures at 7 days. The results that he mentioned in New York related to 4-day cultures. I think that if he is going to look for differences between preimmunized and postimmunized individuals, he must look at 4 days. He must look at a time when a secondary type of response will show up in culture. By 7 days, the reaction between the two cell populations may be at a maximum already. In other words, the cells may have become sensitized in culture. I would look for an increase at 4 days when we find very little or no reaction between cells from nonimmune individuals, as was verified by the destructive ability of the lymphocytes on fibroblasts.

DR. CEPPELLINI: That is a possibility, although to me it is not very likely.

DR. BRENT: I feel quite sure that the work of Drs. Bain and Lowenstein and Dr. Hirschhorn and his colleagues is a major contribution not only to the typing problem but to our understanding of the immunological competence of lymphocytes in general. There are, however, one or two points that I would like to make. Did Dr. Hirschhorn say that the enlarged cells form gamma globulin *in vitro*? Do they, then, form specific antibodies? The second point concerns the apparent symmetry of response in the experiments of Dr. Hirschhorn and his colleagues, and it is also particularly pertinent to the experiments described by Dr. Lowenstein. It seems to me that, if one is interested in this phenomenon as an approach to the typing problem, it is unsound to use two cell types of different genotypes, *both* cell types being capable of producing immunological responses. With that kind of setup I don't see how one can disentangle the response of A to B from that of B to A. It seems to me that the essential requirements of the test are (1) that the lymphocytes be from the future graft recipient, and (2) that the material from the potential donors be immunologically noncompetent cells (macrophages? epidermal cells?), or *dead* blood leukocytes, or cell-free extracts or disintegrates of blood leukocytes—i.e., material which, though antigenic, cannot itself contribute enlarged cells to the mixture.

DR. HIRSCHHORN: As to the first question, about the production of the specific antibody, we are currently working on this. We have some preliminary results that there is production of anti-streptolysin-O when the cells are being stimulated with streptolysin-O. We do get an increase in titer when either streptolysin-O or phytohemagglutinin is used as a stimulant.

On the question of symmetry of response, when we did our first extract experiments, which were the ones reported in Science, by chance the extracts did produce symmetry. This is no longer the case. We are finding cases where the extract of A will produce a different response in B than the extract of B will produce in A. We have similarly found this by actually doing chromosome examinations of the mitotic figures from male-female mixtures, and have found that, while in some cultures we find approximately equivalent numbers of male and female mitoses, in a number of other cultures we find the female or the male to be preponderant.

I would like to make two brief comments. One is that I agree with Dr. Ceppellini that a biochemical test might be useful in quantitating this system. I would, however, prefer not to use uptake of H^3-thymidine as that test for the simple reason that these cells are not truly in synchrony and thymidine uptake is related to the mitotic synchrony of these cells. On the other hand, Dr. Cooper in Bethesda has recently shown a marked increase in reactive cells of uridine uptake, even in the first 30 min of culture, when a stimulant is present. I believe that this might provide us with a better and possibly somewhat cleaner system than thymidine uptake. It may be possible to obtain good quantitation after 24 hrs. or even a somewhat longer time, while the peak of mitoses doesn't occur until well after this period.

DR. LOWENSTEIN: Dr. Brent, thank you very much for your suggestion. We felt that this pilot study was worth doing in order to determine the effect of cross-transplantation upon the mixed leukocyte reaction. I think it is well to point out that the techniques used by Dr. Hirschhorn and his group and our culture techniques differ somewhat. For instance, I believe that in cultures from single unsensitized individuals without the addition of phytohemagglutinin Dr. Hirschhorn finds that at the end of 7 days 5 to 10 percent of the cells are large cells, and that there is a considerable number of mitotic figures. With our technique, we have found in unmixed cultures fewer than 1 percent large cells and only the very occasional mitotic figure present at the end of 5 days. Why this is, I don't know, except that I think possibly the addition of fetal calf serum to the culture medium in Dr. Hirschhorn's technique may be one reason for some of the difference in results. Then, of course, Dr. Hirschhorn arrests mitoses with Vincaleukoblastine. In like manner, we have tried leukocyte extracts and have had the same experiences as Dr. Ceppellini. However, I don't doubt that Dr. Hirschhorn has been able to produce results with leukocyte extracts. It's just that I think his technique differs considerably from ours, and maybe the explanation of this difference in results resides within the differences in techniques. These are the questions we hope to iron out at this workshop.

You will note from Dr. Hirschhorn's figure that his maximum mitoses were found after 7 days of culture, and the maximum numbers of large cells were found after 8 days. Using our technique, we have measured H^3-thymidine uptake in leukocyte mixtures after both 5 and 7 days of culture. The uptake in the

7-day cultures sometimes was increased and sometimes was decreased, compared with the 5-day cultures. None showed a great increase at 7 days, and the mean value at 7 days (46,694 cpm) was almost identical with that at 5 days (44,138 cpm).

DR. MILGROM: In connection with the discussion on using tissue cultures in studies on transplantation immunity, I would like to describe briefly our work on mixed agglutination which is going to be presented at Duke University.

We were interested in the reactions of homotransplantation sera with monolayer cell cultures. In one experimental series, transplantation sera originated from C57BL mice grafted with C3H skin. The reactions were performed on monolayers of L cell line, which are fibroblasts originating from C3H mice. The cell cultures were incubated with transplantation sera and thereafter an indicator system was applied to demonstrate the binding of antibodies to the cell surface. The indicator system was composed of human erythrocytes sensitized by their corresponding antiserum of mouse origin and agglutinated by rabbit antibodies to mouse gamma globulin. Adherence of the indicator erythrocytes to the cell cultures indicated a positive reaction. We obtained consistently positive results starting in the second week after transplantation (Abeyounis, Milgrom, and Witebsky, in press).

A similar procedure was also applied for detecting transplantation antibodies accompanying homograft rejection in rats and rabbits.

The mixed agglutination procedure in cell cultures can also be employed in order to detect the binding of complement. We have not yet performed such experiments with transplantation sera; however, we were successful in elaborating this procedure in using heteroimmune sera. Cell cultures were incubated with the corresponding rabbit antisera and guinea pig complement. The indicator system was composed of tanned erythrocytes coated by guinea pig serum and agglutinated by a rabbit antiserum to guinea pig serum. This indicator system detected the binding of complement but not of antibody (Milgrom *et al.*, 1964).

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Summation

H. Sherwood Lawrence

I now find, as others preceding me have, it was much easier to summarize this conference before rather than after the fact. I would just add a few scattered and perhaps related comments first to remind ourselves that it is still a good biological principle that the genetic disparity between the donor and the recipient of a graft will control the tempo and intensity of graft rejection. Another early principle written by Medawar in his "Uniqueness of the Individual", relates to the undesirability of possession by the homograft donor of antigens not possessed by the recipient. It seems to me that if we try to isolate, identify, and characterize every last histocompatibility antigen, this could be the impossible task that David Newth's figures deal with, as do other genetic estimates of the lack of success that one could expect.. If we are, rather, faced with the problem of bridging this disparity in a small number of antigens between donor and recipient, it may be much less bleak a prospect. Terasaki's observations, although from a selected group, point in this direction.

Related to this is the immunological mechanism of homograft rejection itself. Here the orthotopic homograft which establishes vascular and lymphatic connections with the host is under consideration and excluded are dissociated cell populations as homografts which are particularly vulnerable to the deleterious effects of serologic antibody. To select a few homely comparisons, for instance, the tubercle bacillus is such a foreign material that it is relatively easy to detect its presence in the infected host. One can have a hemagglutinating antibody or other immune responses to tuberculoprotein and yet the tissue damage encountered in the actual disease process is a function of the delayed type of hypersensitivity which cannot be induced by the dissociated components of the bacillus, unless coupled with the lipopolysaccharides. Another example, is diphtheria infection. Seeking antibody to the diphtheria bacillus one may find antibody to atoxic diphtherial proteins, diphtheria antitoxin which will neutralize toxin but may not precipitate in the presence of antigen or univalent, reaginic antibody. Or the response may be associated with delayed allergy to diphtheria toxoid. Yet the disease, a focal point of interest, occurs in relation to the presence of diphtheria toxin which happens to be, by sheer accident, a toxic protein that combines with certain portions of mammalian tissue and causes disease. Another interesting facet of this untangled skein is that diphtheria bacilli are not toxic unless and until they are infected with a particular bacteriophage which causes the organism to secrete the highly specific protein diphtheria toxin.

These examples point up the complexities partially unraveled in relation to known systems and suggest the wisdom of restraint in interpreting observations secured when dealing with unknown systems whose complexities we can only guess at. I wonder if some of the *in vitro* tests are not exploring one unknown with another. I don't think this is at all a bad thing as long as we continue to keep in perspective the possibility that we are doing just that. Particularly appealing in this regard is Winn's finding, designed as a control, that normal sera fixed complement. This sort of observation holds a promise that may lead to some new approach to the problem.

There were many tests detailed in this symposium. I would like to mention a word or two about several. The "third-man" test is fine except one cannot exclude the participation of the immunological apparatus of the man being tested. This test may be revealing hypersensitization of a host rather than indicating histocompatibility sharing of antigens. The finding of platelet agglutinins is of interest in that there is such sharing of antigens by polys, by lymphocytes, by monocytes, and by platelets. As Dr. Merrill and Dr. Amos suggested, it may be the agglutination of platelets that ensures a white graft reaction by interfering with vascularization. This could be related to the fact that one sees the white graft with regularity when skin grafts are used to sensitize humans, only if the latent period between first and second set grafts is a week or less. But if one uses various leukocyte preparations for sensitization of humans

it is very difficult to avoid a white graft. It may be that antibody induced against one part of leukocytes but reacting also against platelets, with resulting platelet thrombi, makes a white graft reaction inevitable. The "white graft," therefore, may be an expression not so much of the quantity of antibody as of the quality.

I wondered if the white-cell agglutinin test and the lymphocyte stimulation test are not deflected from their designed purpose of revealing histocompatibility antigens by the predilection of such cells to combine with or become enveloped by extraneous unrelated antigens and antibodies. Are the cells concerned innocent bystanders coated with an antibody they never made, and may they be coated with the various products or our enteric and other bacteria that we are feeding into our system all the time? This circumstance may have little to do, then, with transplantation immunology, but may just reflect the natural mammalian condition.

In considering immunization procedures to produce leukocyte agglutinins or other antibody reagents to interact with histocompatibility antigens, it is worthwhile to recall the question of how many antigens are possible and the properties of the particular antigen. For instance, streptococcal-M substance is a very poor antigen, compared with pneumococcal polysaccharide; it may therefore make a difference of which antigen one asks a particular question. Again, the route of access, as Dr. van Rood's paper brought out, as well as the dose of antigen and the schedule of administration, will all condition the immune response, as you are very well aware.

A brief word about the *in vivo* mode of testing: this leads one to ask whether the skin graft itself is not the most difficult and forbidding of all tests to measure histocompatibility. Whether the skin graft may, in its behavior, reflect other factors, compared, for instance, with a kidney homograft. Instead of being prey to a series of very small capillaries that nourish the skin, the kidney homograft is in effect an *in vivo* vascular shunt where antigen is poured into the host's system intravenously, there to do what it may. On occasion it may cause sensitization, depending on the dose; on other occasions it may cause desensitization. Desensitization, coupled with Imuran and a few other maneuvers, may result in prolongation of a kidney graft. This may have nothing to do with the behavior of a skin graft. To cite the one outstanding intimation of this possibility: a host accepted a kidney graft successfully and was subsequently grafted with skin from the donor of the kidney. He proceeded to reject that skin graft but this episode had no demonstrable effect on the *in situ* kidney.

These and other mysteries I can only mention but not explain, unfortunately. Also, would it be worthwhile thinking about the fact that the lymphocytes used in the lymphocyte transfer and lymphocyte stimulation tests may bear transfer factor and how this may affect the test? The cells used not only contain antigen but may possess or acquire the effector reagent which has been shown to mediate homograft rejection.

For the above reasons. I would lean more toward certain *in vivo* tests or would rather do the *in vitro* tests in conjunction with the *in vivo* ones in evaluating histocompatibility. I think if there was a weakness in all of the presentations of this morning and this afternoon it was this admittedly extremely difficult counterpart to measuring what happens in the test tube; that is the confirmation with the *in vivo* observation, a safeguard which I think is better done in a prospective than in a retrospective way.

Does the serum antibody in the leukocyte agglutinin test measure antigens that leukocytes happen to have? Does the lymphocyte stimulation test measure a response to antigen which the cells are incapable of doing otherwise? Does the macrophage inhibition test parallel delayed allergy and merely parallel homograft rejection, yet have a variable relationship to *in vivo* reality? Admittedly, all these tests are the best we have available, yet questions do remain to be clarified.

In the best of all possible worlds, then, will the *in vitro* tests reflect *in vivo* events? I would think in the best of all possible worlds this would be the outcome and then the tests would have a great predictive value for the successful exchange of tissues. In the worst of all possible worlds, and this alternative is not catastrophic, we are really defining for other cell populations that which was so exquisitely defined for the red cell antigens. Even this least reward is very important, just as this whole activity as a general biological exercise is of tremendous potential significance.

I would like to conclude on a heretical note; that is, with the question, "Is the homograft response really an actively acquired immune response, or could it be an elevation of an isoimmune state?" I don't know the answer to this, nor whether there can be an answer

forthcoming, but it may be in the methods by which we induce and detect the presence of homograft immunity, that we have an event which could lead us to believe it is actively acquired. I think perhaps in the immunology of the near future the clarification of what isoantigens are and just what isoimmunity is, could be very revealing in respect of the histocompatibility problem. In this connection, it may be helpful for us to remember the disappointment and frustration of the autoimmunologists' finding tissue-specific serum antibodies for which no function could be assigned until Paterson's recent observation that they may exert a protective effect. Perhaps this holds a clue to the normal state of affairs in regard to our own tissues and histocompatibility. The circulation of such serum antibody may serve some homeostatic purpose to inhibit one from treating one's tissues as if they belong to someone else. The additional reality of the enhancing antibody of Kaliss and enhancing antibodies that have been shown to alter skin homograft survival make the extrapolation to the homograft problem and histocompatibility testing a potentially productive application of this viewpoint.

Part II

WORKSHOP ON HISTOCOMPATIBILITY TESTING

edited by

D. Bernard Amos

Technique for Demonstrating Leukocyte Agglutination

J. Dausset

1. Preparation of Sera

The sera having been separated in the usual way, sodium azide is added, to a final concentration of 1:1000. The sera are kept frozen at −20°C until required. They are inactivated by heating for 30 min at 56°C before use. The sera to be tested, the normal control sera (from nontransfused male), are treated in the same way. Infected, hazy, or fatty sera are either discarded or made clear by filtering or centrifugation at high speed (10,000 g).

2. Technique Using Defibrinated Blood

a. *Preparation of the leukocyte suspension.* Ten milliliters of blood compatible in the ABO group system with the serum to be tested are collected in a 100-ml Erlenmeyer flask containing 10 glass beads. The blood is immediately defibrinated by hand circular shaking (approx. 150 rotations/min in a horizontal plane, with a diameter of rotation of approx. 5 cm). After shaking for 10 min, the blood is separated from the fibrin clot and poured into a siliconed test tube (14 × 1.6 cm).

One quarter of its volume of high-molecular-weight dextran is added to the defibrinated blood.* Mixing is ensured by three inversions of the tube. The latter is then inclined at 45 deg. After sedimentation for 25 min at laboratory temperature, the tube is stood vertically for 10 min. The supernatant, carefully pipetted off with a Pasteur pipette, is the leukocyte suspension and consists, on an average, of 3,000 to 4,000 leukocytes/mm³. This suspension is kept at 4°C in siliconed tubes until used. The interval between collecting the blood and beginning the test must not exceed 3 hr.

b. *Test.* In a nonsiliconed test tube (5 × 0.6 cm) are placed 2 drops of inactivated serum to be tested or its dilution in saline. Similar tubes are prepared with inactivated normal control sera. To each tube is added a drop of the leukocyte suspension.

After incubating for 1½ hr at 37°C, most of the supernatant is discarded with a Pasteur pipette, one drop of 5-percent acetic acid is added to each tube, and the tube is gently shaken. The hemolysis of the few remaining red cells is complete within a few seconds. A thick drop of the mixture is then put on a slide and examined under the low power of the microscope (ocular 8 and objective 7 in a binocular microscope).

If the reaction is negative, the leukocytes are evenly spread out. If the reaction is positive, clumps of leukocytes are formed which are scored as follows: +, ++, +++, or ++++.

3. Technique Using Blood Treated with EDTA

a. *Preparation of the leukocyte suspension.* Nine milliliters of blood are collected into 1 ml of a solution of 5-percent sequestrene (EDTA) in 0.15M NaCl. Separation of leukocytes with dextran is carried out as above except that the tube is kept inclined for 20 instead of 25 min and that the suspension must be adjusted to 6,000 leukocytes/mm³.

b. *Test.* The reading can be made as above; however it is better to use an inverted microscope (ocular 8, objective 7) in order to read the reaction in the tube without adding any acetic acid. Scoring of the results is carried out as described above.

* A preparation of dextran with a molecular weight of 160,000 (Poviet Production N. V. Maurits Kade 14—Amsterdam) is dissolved in 900 ml of 0.8-percent NaCl. For complete solution it is necessary to heat to boiling point. After cooling, the volume is made up to 1,000 ml by adding 0.8-percent NaCl. The solution thus obtained is filtered with sterile precautions through a Karlsson or Seitz E. K. S. filter and kept sterile.

Dextran grade A (molecular weight: 200,000 to 300,000) from Mann Research Lab. can also be used.

4. Technique Using Phenol

The test is performed with one drop of leukocytes suspended in EDTA plasma, one drop of inactivated serum, and one drop of 0.2-percent phenol in saline (prepared from a stock of saturated phenol—preserved in a dark fluid—diluted just before use at 1:30).

An Agglutination Technique for the Demonstration of Leukocyte Isoantigens in Man

R. Payne

The method described below for the identification of leukocyte isoantigens in man is essentially a modification of the technique originally developed by Dausset.

1. Reagents

(1) Acetic acid (3-percent).

(2) Physiologic saline (0.9-percent).

(3) Sodium hydroxide (0.1 N).

(4) Red-cell typing sera: anti-A, anti-B, and anti-D; other typing sera as required for the special selection of leukocyte donors of specific red-cell groups.

(5) Red-cell sedimenting solution: polyvinylpyrrolidone (PVP) (4-percent) in saline. PVP dissolves more rapidly at 37°C. Adjust pH to 6.8 to 7.2 with 0.1 N NaOH. Sterilize in autoclave at 15 lbs for 20 min. Store in refrigerator at 4 to 6°C. The solution is stable and will keep indefinitely. PVP Type NP-K30 is available from Antara Chemicals (Division of General Aniline and Film Corp.) with offices in New York, Chicago, and San Francisco.

2. Glassware and Equipment

One-milliliter, 5-ml, and 10-ml serologic pipettes; Pasteur-type capillary pipettes; glass beads, 4 mm; 600-ml beakers; 125-ml Erlenmeyer flasks; test tubes without lip, 12 x 75 mm, 16 x 150 mm; test-tube racks; Spencer Bright Line Improved Neubauer Blood Cell Counting Chamber with red and white blood cell pipettes; microscope slides, 3 x 2 in.; two waterbaths, 37 and 56°C; centrifuge; microscope; 2-ml Boerner centrifuge filters with Seitz pads; sterile 30-ml syringes and #20 gauge hypodermic needles.

3. Preparation of the Leukocyte Suspension

a. *Collection of blood.* Obtain blood from donors by venipuncture. (For details regarding red-cell blood groups, see Sec. 4.) Defibrinate blood in an Erlenmeyer flask with five glass beads for 20 to 30 ml of blood. This will provide leukocytes for approximately 50 tests. Smaller quantities may be used but less than 10 ml of blood does not permit adequate separation of leukocytes. Gently rotate the flask for 10 to 15 min, until a firm clot forms around the beads. Blood treated in this way will be essentially free of platelets.

b. *Sedimentation of the blood.* Separate the red cells from the leukocytes by sedimentation with PVP solution. Mix by repeated inversion 16 ml of defibrinated blood with 4 ml of PVP solution (ratio 4:1) in a 20-ml test tube. Remove surface bubbles, which may trap red cells. Set the tube of blood and PVP at a 45° angle in a 600-ml beaker. Place in a 37°C water bath. Settling of the red cells will require from 20 to 50 min, depending on the sedimentation rate of the particular blood sample. The supernatant leukocyte-enriched plasma should be removed when it appears turbid with a faintly pink tinge. Withdraw the supernate carefully with a Pasteur pipette so as not to disturb the underlying red-cell mass. The number of residual red cells in the supernate should not exceed the number of white blood cells. If sedimentation has proceeded for too long a period, the supernate will be clear and the leukocytes will have precipitated into the red-cell portion.

c. *Standardization of the leukocyte suspension.* After thorough mixing determine the number of leukocytes in the suspension by the routine clinical method. When prepared as outlined above, the usual suspension will contain from 5,000 to 10,000 white blood cells/mm³. These will be made up of approximately equal numbers of granulocytes and lymphocytes. The number employed in the agglutination test may vary between 3,000 and 4,000 cells/mm³. If no more than this number of cells is harvested it is ready for use. Ordinarily the suspension will require dilution.

The diluent is prepared by centrifuging the sedimented red-cell portion which remained after removal of the leukocytes. The clear serum-PVP supernate is taken off, heated for 30 min at 56°C, and cooled before use. Heat inactivates a thermolabile inhibitor of leukocyte agglutination, presumably complement. The amount of active inhibitor contributed by the cell suspension to the final test mixture will not prevent agglutination. Heat-inactivated serum, either from the leukocyte donor or from a red-cell-compatible person, may be substituted for the above diluent. Avoid using sera for diluent originating from multitransfused patients or recently gravid women, as these may contain leukoagglutinins.

d. *Stability of the suspension.* Leukocytes must be used on the day of preparation of the suspension, preferably within a few hours of collection. If there is a lapse of time between preparation and use, the suspension should be kept in the refrigerator (4 to 6°C). These precautions tend to prevent spontaneous aggregation of the cells.

4. Leukocyte Antisera

a. *Source, collection, and preservation.* Sera containing leukocyte agglutinins must be collected from recently gravid women who have had at least two pregnancies. The serum from about 10 percent of women with four or more pregnancies will contain these antibodies. These may be detected by testing the woman's serum against the leukocytes of her husband. The specificity is established by testing the serum against a panel of leukocytes. Because white blood cells must be used on the day of venipuncture, the individual investigator must set up his own panel. For this purpose, it is convenient to use persons of red cell group O, thus avoiding the interference of red cell agglutinins, anti-A and anti-B. The leukocytes from 10 unrelated donors collected at random could constitute a panel which would include most of the isoantigens. Leukocyte agglutinins obtained from multitransfused patients will not be suitable, since their activity closely resembles a panagglutinin.

The need to work with undiluted sera (titers are usually low) makes it advisable to take a pint sample at a single bleeding. The serum is inactivated by heating at 56°C for a half hour. It is stored in the frozen state (−20°C), in which condition the agglutinin appears to be stable for years. Immediately prior to use of the serum in the agglutination test, it should be examined for turbidity. If debris is visible, an aliquot of serum should be clarified by passage through a 2-ml Boerner-type centrifuge filter.

b. *Problem of red-cell agglutinins in leukocyte antisera.* Inasmuch as the usual suspension of leukocytes will contain red cells and most antisera will contain the "naturally" occurring red-cell isoagglutinins, leukocyte suspensions whose red cells are inagglutinable by the test serum must always be used. Otherwise leukocytes will adhere to the red-cell agglutinates, thereby giving rise to doubtful reactions. Red-cell agglutinins of immune origin may interfere in the same manner. For this reason the red cells of donors of antileukocyte sera must be typed (ABO and Rh). The antisera must also be screened for the presence of red-cell agglutinins of immune origin. Two procedures may be used to prevent the interference of red-cell agglutinins: (1) absorption of the red-cell agglutinins from the test serum, or (2) use of a leukocyte suspension whose red cells lack the antigen. The latter is the preferred method, as it requires less effort and circumvents any loss of white-cell agglutinin activity which may occur during absorption. The utilization of leukocytes from a blood group O donor prevents interaction with sera containing anti-A or anti-B; an Rh negative donor will do the same for sera containing anti-D. These are the most common red-cell agglutinins that will be encountered.

The absorption procedure should be utilized in the preparation of leukocyte antisera for studies of families whose members are of differing ABO blood groups. Absorption is carried out as follows: Prepare defibrinated blood of red cell groups A and B for sera of group O, group A for sera of group B, and group B for sera of group A. Remove the leukocytes from the defibrinated blood as in preparation of a leukocyte suspension, *except* do not allow the red cells to settle as long. By this maneuver, fewer leukocytes will sediment into the red-cell portion. Wash the red-cell mass with three volumes of normal saline. Repeat three times, packing the cells firmly by centrifugation for 20 min at approximately 2,500 g between washes. After each wash, remove, along with the supernatant saline, the upper cell layer which will still contain leukocytes. For absorption, use the most firmly packed red cells from the bottom of the tube. This technique minimizes both dilution with the saline and absorption by residual leukocytes, which is vital because of the usually low titer of leukocyte

antisera. Absorb one volume of serum with one-half volume of red cells. Absorption temperature should coincide with that for optimal activity of the agglutinin being absorbed. Incubate for ½ hr. Mixing frequently will increase the efficiency of absorption. Two or three absorptions are sometimes necessary.

Neutralization of anti-A and anti-B with Group Specific Substance in solution has not been consistently effective. The volume required frequently dilutes out the leukocyte antibody activity. Trials with dried powder prepared from the solution showed this material had an inhibitory effect upon leukocyte agglutination.

5. Leukocyte Agglutination Test

(1) In duplicate 12 x 75 mm test tubes, add 0.05 ml (1 drop of leukocyte suspension to 0.15 ml (3 drops) of specific inactivated antileukocyte serum.

(2) Mix by gently shaking and incubate in a 37°C water bath for 90 min.

(3) Add 0.15 ml (3 drops) of 3-percent acetic acid to each test mixture. Mix by gentle shaking to facilitate red-cell lysis. Allow 2 min to resettle.

(4) Pick up the deposited cells from the bottom of the test tube with a Pasteur pipette and transfer a large drop onto the surface of a 2 x 3 in. alcohol-cleaned microscope slide. The slide conveniently holds six tests.

(5) Before examining under 100 X magnification, allow the cells to settle to the surface of the slide.

In a negative test, the leukocytes will be evenly dispersed throughout the field. Small clumps of three or four aggregated cells will be occasionally observed.

In a positive test, the number of cells in the agglutinated clumps will be variable. The degree of agglutination can be graded as ++, +++, or ++++. Small aggregates of leukocytes with an equal number of free cells throughout the fields are recorded as ++, and large massive clumps with few, if any, isolated leukocytes are recorded as ++++. A single mass of tightly agglutinated cells comparable to reactions with commercial red-cell typing antisera is not produced.

6. Sources of Error

(1) A common source of error in reading the tests is the removal of floating nonagglutinated cells rather than the deposited agglutinates. If readings on duplicate test tubes do not agree, or if very weak, doubtful agglutination is observed, an additional drop of cells from the same tubes should be removed and examined.

(2) If the leukocyte suspension is prepared from agranulocytic donors, negative results will be obtained because pure lymphocyte suspensions do not appear to be agglutinable. A suspension prepared from healthy donors may contain only lymphocytes if the sedimentation has been prolonged.

(3) Cell suspensions prepared from donors with infection and elevated leukocyte counts exhibit a tendency to produce loose aggregates. These white blood cells will give rise to weak false-positive readings.

(4) Prolonged incubation of the test mixture for more than 3 hr will produce spontaneous aggregation of the leukocytes.

(5) The ready adherence of white blood cells to microscopic debris will make reading of the results difficult. The following kinds of debris are frequently encountered: (a) minute fibrin strands in serum from freshly clotted blood, (b) flocculent precipitates of denatured proteins in frozen sera, and (c) detritus from improperly cleaned equipment. The precaution of filtering sera immediately prior to use cannot be overemphasized in avoiding false-positive tests attributable to debris.

(6) The failure to take into account the presence of red-cell agglutinins may result in false-positive readings. Leukocytes will adhere to the red-cell clumps or stroma in the absence of specific leukocyte isoantigen.

(7) The occurrence of red-cell rouleaux in the test drop will also make interpretation of the reading difficult. Their occurrence indicates failure to add sufficient acetic acid for lysis of the red cells.

7. Evaluation of the Method

The method described above is, on the whole, relatively easy to learn and requires only the usual laboratory equipment. There are, however, some special problems regarding the leukocyte antisera which should be

mentioned. It might be inferred from the description that each serum obtained from a recently gravid woman contains an agglutinin specific for a single antigen, whereas a serum obtained from a multitransfused individual identifies several antigens. While the first may be true in certain instances, it has not been clearly established and there is some evidence to the contrary. A few leukocyte antisera from parous women appear to contain agglutinins which are separable by cross-absorption. Nevertheless, sera from this source are the best that are presently available. Another point which should be kept in mind is that, while many leukoagglutinins give consistently reproducible reactions, results with others are less reliable. A multiplicity of different antileukocyte sera, i.e., with varying specificities, has been collected from parous women. One cannot rely on finding duplicate antisera readily. Occasionally duplicate types from new women will be encountered in the process of serum collection. For this reason it is important to collect a given serum in large quantity initially.

The tendency of leukocyte suspensions spontaneously to form small aggregates on storage for more than a few hours is a serious handicap. In these circumstances, the antigen cannot be prepared in advance for later use. The instability of the leukocyte suspensions now employed permits neither the exchange of cell samples between investigators for verification of results nor the establishment of a central reference panel. The development of a method for the preservation of leukocytes or their antigens will make a significant advance in this field of work.

An Agglutination Technique for the Demonstration of Leukocyte Antigens in Man Using Leukocytes from EDTA Blood

J. J. van Rood and A. van Leeuwen

1. Selection of the Sera

The calculations necessary for the selection of the sera which might recognize identical or allelic leukocyte isoantigens have been given before (van Rood and van Leeuwen, 1963).

Sera were stored at −20°C after the addition of sodium azide (1 drop of a 10-percent solution per 8 ml of serum). When necessary, the sera were cleared by centrifugation at 16,000 g for 10 min. Before use, the sera were inactivated by heating at 56°C for 20 min. Infected sera or sera which are not completely clear will give false-positive reactions.

2. Leukocyte Suspensions for the Agglutination Tests

Blood was obtained by clean venipuncture. About 8 ml was collected in a siliconized centrifuge tube containing 1 ml of 5-percent EDTA in physiological saline. After mixing, the EDTA blood was poured into a siliconized tube containing 2.5 ml of 5-percent dextran (mean molecular weight: 200,000) in physiological saline. Foam was removed. The tube was incubated at 37°C for 30 min at a 45° angle. After incubation, the upper four-fifths of the supernatant plasma was transferred to a siliconized centrifuge tube with a siliconized pipette and the leukocytes were counted. This part of the plasma, which contained 3,000 to 9,000 leukocytes/mm³, was used for the agglutination reaction. Care has to be taken that during removal of the leukocyte suspension the tube remains in the same 45° angle position. If this is not done, the narrow band of red cells in the supernatant plasma present on the under side of the tube will be whirled up and will contaminate the leukocyte suspension. Leukocytes from defibrinated blood were obtained in the same manner, except that 1 ml of 2-percent Tween-80, in buffered physiological saline, was added to 2 ml of the dextran solution. The leukocyte suspensions were used as soon as possible, but never more than 2 hr after the venipuncture.

3. Agglutination Reaction

Two drops (approx. 0.10 ml) of inactivated serum or serum dilution were mixed in nonsiliconized, round-bottomed tubes (50 x 7 mm "critical"!) with 2 drops of a leukocyte suspension, if the number of leukocytes was between 8,000 and 13,000/mm³. If the count was between 6,000 and 8,000, 3 drops were used, while 4 drops were taken with counts of less than 6,000 and 1 drop with counts of over 13,000/mm³. The relation between the number of leukocytes in the suspension and the score obtained is illustrated in Fig. 1. The tubes were then incubated for 105 (maximally, 120) min at 37°C in an incubator, not a waterbath. After the incubation, the supernatant plasma was removed, and one drop of 6-percent acetic acid was added to the sediment to lyse the red cells. The sediments were transferred to a nonsiliconized slide and, without spreading them, observed microscopically (magnification 50 to 100x). False-negative results can be obtained if the clumps are removed with the supernatant plasma. This can quite easily be done, especially when tubes with a diameter of more than 7 mm are used. Sometimes extra acetic acid has to be added because the red cells are not completely lysed. This can be done on the slide. All glassware should be meticulously clean.

To facilitate microscopical readings of the leukocyte agglutination reaction, the following device was constructed. From ordinary glass a large microscopic slide, 50 x 300 mm, was made. Lines were cut in the glass with a glass knife, dividing it into 20 equal rectangles. The ordinary object-holder of the microscope was removed and replaced by a light-metal strip, 130 x 70 mm, with a rectangle in the center of 30 x 50 mm. Two springs made it possible to secure the large microscope slide, lightly clamped, on the

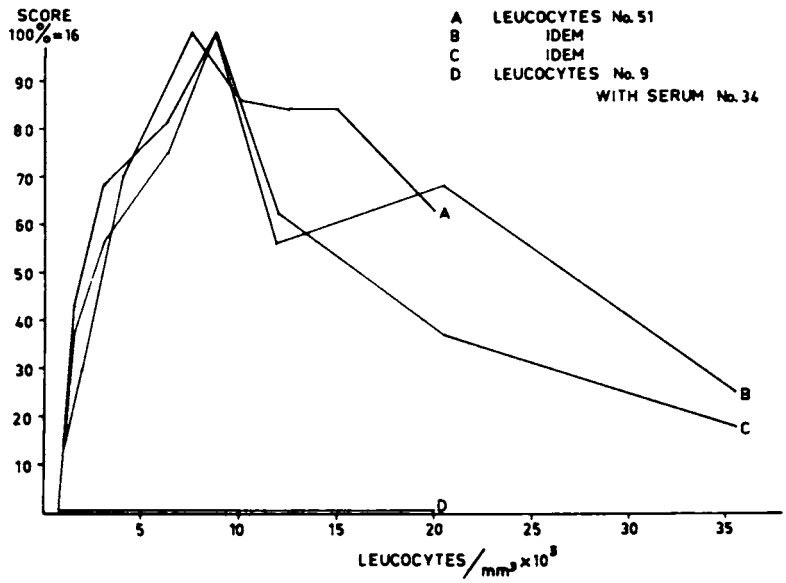

Figure 1.—Relationship between number of leukocytes per mm³ in the cell suspension and the score obtained using leukocytes from EDTA blood. The serum-leukocyte combinations A, B, and C were incompatible; combination D served as a negative control.

metal strip. In this way, the use of the mechanical stage was still possible, while 20 samples could be read without changing the slide (Fig. 2).

Titration of the serum was performed by stepwise twofold dilution in saline. When leukocytes from defibrinated blood were used, essentially the same procedure was employed, with the single difference that normal inactivated AB-serum was used as the diluent. In this test, the number of leukocytes in the suspension should not be over 6,000/mm³, because with higher counts false-positive reactions do occur.

When the results of the EDTA test were equivocal or negative, especially when sera were used that were known to give false-negative agglutination reactions, absorption tests were done.

4. Leukocyte Suspensions for the Cross-Absorption Experiments

These suspensions were prepared as described for the agglutination experiment. "Packed" leukocytes were used for the absorption, i.e., the leukocyte suspension was centrifuged for 5 min at 16,000 g, and the plasma was removed. It is essential that the sediment is really "dry"; otherwise, a dilution effect will mimic absorption. The cell suspensions contained not only leukocytes, but also erythrocytes and platelets. The erythrocytes do not contain the leukocyte isoantigens under study, in contrast to the platelets (Dausset, 1959; van Rood, van Leeuwen and Eernisse, 1959). As these antigens are present on the platelets only if they are also carried by the leukocytes, the contamination of the cell suspension with platelets did not introduce a complication. The average suspension contained 100 to 300 erythrocytes and 2,000 to 10,000 platelets per 100 leukocytes. An average of 2.5 x 10^8 leukocytes could be isolated from 70 ml of blood.

5. Cross-Absorption Experiments

Samples containing 2.5 x 10^8 packed leukocytes were obtained from at least 10 donors. For most sera, this number of leukocytes will be in most instances sufficient to absorb the agglutinins of 1 ml of serum. Each sample was incubated for 30 min at 37°C in a waterbath with 1.0 ml of inactivated serum containing antibodies against leukocytes; the tubes were shaken at 10-min intervals. After centrifugation of the mixture at 16,000 g for 10 min, 2 drops of the absorbed serum were tested against leukocytes of the donor whose

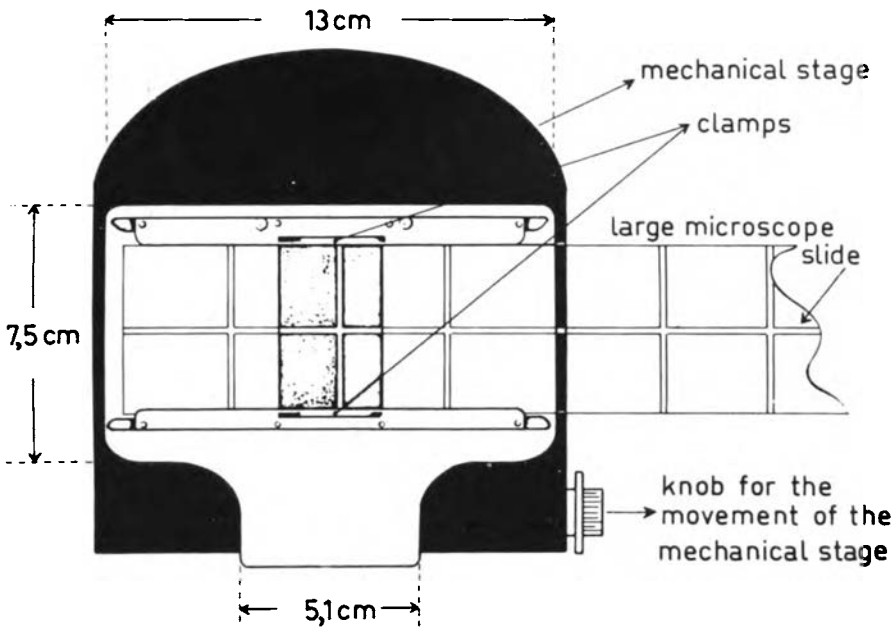

Figure 2.—Bird's-eye view of the device facilitating the microscopical reading.

cell suspension had been used for the absorption, and further against the leukocytes from the other donors. To exclude the possibility that the absorption of the agglutinin was caused by a nonspecific effect, the serum was absorbed not only with cells that carried the corresponding antigen, but also with cells that did not carry this antigen.

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Agglutination of Leukocytes

Parviz Lalezari

1. Preparation of Leukocyte Suspension

A 15-ml sample of blood is collected in a 125 x 15 mm siliconized test tube which contains 0.15 ml of 10-percent Di-sodium EDTA. An additional 10 ml of blood is also collected in a nonsiliconized tube containing 0.1 ml of 10-percent EDTA. From this sample, plasma is separated and saved.

The siliconized tube is centrifuged at 350 g for 15 min in a swinging-head table centrifuge. The centrifuge is stopped slowly, using an additional attached rheostat. By this centrifugation, red cells and white cells are sedimented, but platelets remain in suspension. The platelet-rich plasma is transferred to a nonsiliconized tube and centrifuged at 1350 g for 20 min. This will provide platelet-poor plasma. The platelet-poor plasma thus obtained is mixed with the original siliconized tube which contains the sedimented red cells and leukocytes. To this 0.3 ml of 1-percent Polybrene* solution is added and the tube is inverted 10 to 15 times, then permitted to stand at room temperature for about 30 min. The tube is centrifuged at 15 g for 5 min, after which the test-tube wall is examined for the red-cell sedimentation line. The tube is again placed in the centrifuge, but in such a manner that the red-cell sedimentation line is located distal to the centrifuge center, and the 5-min centrifugation is repeated at 15 g. This procedure is repeated until no red-cell line is seen. Usually two or three repeat centrifugations are required.

The leukocyte-rich supernatant is transferred to a nonsiliconized test tube, and the leukocyte count determined and adjusted to 1000/mm³. Then 0.1 ml of 10-percent EDTA is mixed with each 0.9 ml of adjusted leukocyte suspension. These cells then are ready to be used as antigens. The cell suspension thus obtained may be kept at room temperature and used throughout the day.

2. Leukocyte Agglutination Test

Serial double-fold dilutions of the test plasma are prepared in 0.1-ml volumes in 10 x 75 mm nonsiliconized test tubes, using isotonic NaCl solution as diluent. Each test tube receives 0.05 ml of the antigen suspension; they are mixed, covered by parafilm, and incubated at 37°C. As a control, serial double-fold dilutions of the plasmas obtained from the leukocyte donors are prepared and incubated with the cell suspensions.

3. Reading the Results

The results are read after 5 hr and again after 18 hr. The endpoint is always determined after prolonged incubation.

a. *Macroscopic reading.* The agglutinated cells are seen as floating white particles. The nonagglutinated cells form a homogeneous "film" adherent to the test-tube bottom, and thus leave a clear supernatant.

b. *Microscopic reading.* Following macroscopic evaluation, the test tubes are gently mixed and a drop of cell suspension is placed on a slide. The reaction is examined under a dim-light microscope. When almost all the cells are agglutinated in one or a few large clumps, the reaction is called 4+. The aggregation of about 75 percent of the cells represents 3+ and the aggregation of half of the cells represents a 2+ reaction. In a 1+ reaction about 25 percent of the cells are agglutinated. In negative reactions no white-cell aggregates are seen.

CAUTION: On prolonged incubation and only in the presence of high plasma concentration, some degree of cell distintegration may occur. Therefore the results of the first tubes of serial dilutions may be stronger after 5 hr of incubation.

The glassware used in these experiments must be cleaned by brushing with detergents followed by repeated rinsings with tap and distilled water.

* Lots 718–3642, 728–3577, and 734–3620, Abbott Lab., North Chicago, Ill. Suitability of other Polybrene lot numbers for leukocyte separation must be determined before their routine use.

Leukocyte Agglutination Test

Henry E. Wilson

Fresh leukocyte suspensions are employed each day. Approximately 15 ml of blood are collected in a tube containing 0.5 ml of 5-percent disodium versenate and 1.0 ml of 6-percent dextran in physiological saline. Siliconized glassware is used throughout. The blood is placed in three 15 x 200 mm test tubes and allowed to settle for 1 to 2 hr at room temperature, after which the plasma suspension of platelets and leukocytes is removed by Pasteur pipette. The suspension is placed in a 12-ml centrifuge tube and centrifuged at 750 rpm for 15 min, after which the plasma-platelet supernate is removed and the packed leukocytes are resuspended in physiological saline. The leukocytes are washed twice by repeating this procedure and are finally resuspended in physiological saline containing 2.5-percent bovine albumin to a concentration approximating 15,000 leukocytes/mm^3. (One learns to estimate the density visually and adjust dilutions appropriately with a little practice.) A moderate degree of erythrocyte contamination does not interfere with the test.

The agglutination test is performed in nonsiliconized 10 x 75 mm culture tubes. All sera are inactivated in a 56°C waterbath for 30 min prior to testing. Using a capillary pipette, the test serum is double-diluted in physiological saline in 4-drop quantities, starting with a 1:8 dilution.* After completion of the diluting procedure, 0.1 ml of the leukocyte suspension is added to each tube. The tubes are shaken briefly, but vigorously, by hand and placed in a Dubnoff-type shaking waterbath at 37°C for 30 min after which they are allowed to stand at room temperature for 1 hr before reading. The test is read for macroscopic leukocyte clumping with the tube held over a concave mirror in the beam of a standard high-intensity microscope lamp. Positive and negative controls are included in each test. The negative control is set up with a carefully chosen and tested pool of normal sera.

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* In the routine screening of sera, only dilutions of 1:8 and 1:16 are used and the procedure is performed as follows: 7 drops of physiological saline are added to those tubes which will contain the lower dilution and 2 drops of saline to the higher-dilution tubes. It is practical to test approximately 20 individual sera in a single screening series. One drop of test serum is added to one tube in the lower dilution. After mixing serum and saline with the capillary pipette, the entire volume is taken up and 4 drops of the mixture are returned to the tube. Two drops are then added to the higher-dilution tube and the remainder of the serum suspension in the pipette is discarded.

Leukoagglutination Technique*

D. Bernard Amos and Nancille Peacocke

1. Preparation of Glassware

Pyrex tubes used for the collection and processing of the cells are cleaned in chromic acid, rinsed repeatedly, and then siliconized with Siliclad.** Syringes are cleaned in Alconox, and then siliconized; the 6 x 50 mm tubes used for the test are difficult to clean and rinse adequately, so are used only once. The new tubes are washed in distilled water, siliconized with Siliclad, and rinsed, and are discarded after use. The cleaning of the tubes is critical. Unclean or improperly siliconized tubes have resulted in clumping of cells in control tests with normal serum.

2. Collection of Blood and Processing of Cells

(1) Ten milliliters of venous blood are collected into a 16-mm OD tube containing 1 ml of 5-percent Na_2EDTA. Then 2.5 ml of 3-percent Plasmagel*** gelatin are added and mixed with the blood. The tube is allowed to stand at room temperature for 20 to 30 min, by which time most of the red cells have formed rouleaux and settled, leaving most of the white cells and platelets in suspension.

(2) The supernatant, together with the topmost layer of red cells, is removed. This mixture, containing about 14,000 leukocytes/mm^3 and 140,000 red cells/mm^3, is placed in two 10-mm OD tubes and centrifuged in an International Clinical Centrifuge with angle head at between 83 and 100 g for 10 min. The leukocytes and red cells, with some platelets, form a loose button. All but about 0.1 ml of the supernatant plasma containing most of the platelets is removed, placed in a separate tube, and centrifuged at 4,000 g for 10 min, to throw down the platelets and to yield a clear, platelet-free plasma. The platelets are discarded or saved for absorption purposes.

(3) The original cell button is resuspended in the 0.1 ml of plasma left in the tube, and the suspensions are transferred to a 6 x 50 mm tube, mixed well, and allowed to resediment for 20 to 30 min. Because of the high concentration of red cells in this suspension, rouleaux formation occurs and the great majority of the red cells sediment rapidly, leaving the leukocytes and most of the remaining platelets in suspension. This supernatant suspension is transferred to a 10 x 60 mm tube and diluted with saline in the proportion of two or three parts of cell suspension to one part of buffer. This reduces the specific gravity of the suspending plasma.

(4) Approximately 2.0 ml of platelet-free plasma from step (2) are placed in a 7 x 100 mm ampoule.**** The leukocyte suspension from step (3) is layered on top of the column of platelet-free plasma and the tube centrifuged at 100 g in a swinging bucket head for 1 min. The leukocytes pass through the gradient and form a very loose pellet; the platelets remain above the column of undiluted plasma. The speed and time of centrifugation need careful adjustment.

(5) The supernatant plasma, together with the platelet layer, is removed to within about ½ in. of the bottom of the tube. The purified leukocyte suspension is mixed with the remaining plasma, and diluted to a final concentration of 6 x 10^6/ml in a mixture of platelet-free plasma and EDTA phosphate buffer. The concentration of plasma does not appear to be critical between the range of one part plasma to three parts buffer and three parts plasma to one part buffer. Some plasma in the final suspension is necessary to preserve the cells in good condition and prevent nonspecific clumping. The buffer consists of 2.6 g Na_2HPO_4, 3.0 g Na_2 EDTA, and 8.5 g NaCl per liter.

*Aided by USPH grants A1 K6 18399 and GM 10356A1.
**Clay-Adams, Inc., New York, N.Y.
***Laboratoire Roger Bellon, Seine, France.
****Edwards High Vacuum, Niagara Falls, N.Y.

3. The Test

The test is performed in 6 x 50 mm siliconized tubes. As explained above, it is difficult to wash tubes of this size efficiently and new tubes are siliconized and used only once. Although as little as 0.015-ml amounts can be used, 0.025-ml volumes are used as a routine.

The test serum is diluted serially in EDTA phosphate buffer containing 1.3 g Na_2HPO_4, 1.5 g Na_2EDTA, and 8.5 g NaCl per liter (i.e., 100 ml of buffer from the previous section are diluted with 100 ml of saline for use in the test). It is essential to test various concentrations of antiserum; some strong sera show a marked prozone, and some weak sera react only when tested undiluted.

An equal volume of cells prepared as above is mixed with the serum dilutions and the mixture incubated for 2 hr at room temperature. The suspension is mixed once with a capillary pipette, and a drop is transferred to a 3 x 2 in. or 3 x 4 in. microscope slide and read microscopically without the addition of acetic acid. Controls with known negative sera are included with every test and additional controls with known positive sera are run with most tests.

4. Specific Points

Leukocyte agglutination, like tanned-cell agglutination, can give consistent results if a standard technique is carefully followed. Apparently trivial departures from any of the procedures described can result in loss of sensitivity or false agglutination. The cleaning and siliconization of the glassware, speed of centrifugation, concentration of the buffers, and the presence of adequate concentrations of EDTA appear to be extremely critical. Higher concentrations of EDTA are strongly inhibitory, and lower concentrations lead to nonspecific clumping. Some of these points have been discussed in greater detail elsewhere (Amos and Peacocke, 1964).

5. Conclusion

The preparation of the cell suspension sounds complex, since three distinct steps—sedimentation, resedimentation, and passage through a gradient—are involved. In practice, the excellence of dispersion of the leukocytes and the comparative freedom from contamination with platelets and red cells makes the test easy to read. This, together with the freedom from nonspecific clumping, adds to the sensitivity of the test, since weak reactions can be determined with greater confidence.

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Indirect Antiglobulin Consumption Test for the Detection of Human Antibodies Against Skin Cells, Leukocytes, and Platelets

J. Colombani, M. Colombani, and J. Dausset

This test can be used when the antigen is insoluble or carried by insoluble particles. The fixation of the antibody to the antigen is shown by the capacity of the sensitized antigen to absorb (consume) an antiglobulin serum. This absorption is measured by the fall in the antiglobulin serum titer tested against RBC sensitized by an incomplete antibody (indicator system). In the case of human antibodies, the antiglobulin serum is an anti-human globulin serum (A.H.G.) and the indicator system is human O Rh-positive RBC sensitized by an incomplete anti-Rh antibody.

The general performance of the test will be described first, and then the specific details for testing the several types of antigens.

1. The Indicator System

Type O Rh-positive RBC are washed three times in saline; 0.2 ml of packed cells is mixed with 2 ml of an incomplete anti-Rh serum optimally diluted and preheated at 37°C. The sensitization is performed for 1 hr at 37°C. The suspension has to be shaken every 15 min. The sensitized cells are then washed four times in saline and resuspended in 5 ml of saline.

2. A.H.G. Titration

Twofold dilutions of A.H.G. in saline are made in 10 x 75 mm tubes. To 0.1 ml of each dilution, 0.05 ml of the sensitized RBC suspension is added. The tubes are shaken and the mixture is allowed to settle at room temperature for 1 hr. The tubes are spun down for 1 min at 3,000 rpm. The reading is taken macroscopically. The A.H.G. serum is then diluted, so as to contain 256 agglutinating units in 0.1 ml.

3. Antigen Sensitization and Washing

A suitable amount of antigen suspension (as determined by previous trials) is mixed with 1 ml of the serum to be tested. The antigen suspension and the serum are preheated at 37°C before mixing. The sensitization is carried out at this temperature for 1 hr with frequent shaking.

The sensitized antigen is then washed six times in saline. During the washing, the temperature should not rise above 20°C. It must not fall below 15°C during the first two washings. After decanting the supernatant from the last washing, the packed sediment is spun down again and the last drops of supernatant are decanted.

4. Absorption of A.H.G. with the Sensitized Antigen and Titration of the Supernatant

The button of sensitized antigen is then suspended in 0.1 ml of saline and 0.1 ml of suitably diluted A.H.G. (256 agglutinating units) is added. This is left at room temperature for 10 min with continuous shaking. The tube is then centrifuged and the supernatant removed. Serial twofold dilutions of the supernatant are made out to 10 tubes, each tube containing 0.1 ml; 0.05 ml of sensitized type O Rh-positive RBC are then added to each tube and the test is completed in the same way as for the original A.H.G. titration.

5. Interpretation of the Results

Negative control sera are tested simultaneously with the serum under investigation. The results are considered valid if the tests with the negative controls show no more than one tube dilution fall in the A.H.G. titer, when compared with the original A.H.G. titer. The result is considered positive if the test with the serum under investigation shows a drop of the A.H.G. titer of at least two tube dilutions, when compared with the A.H.G. titer of the negative control tests.

6. Platelet Antigen

Nine parts of blood are drawn into one part of disodium ethylenediaminetetraacetate solution (Na2 EDTA 5-percent in saline). To obtain the platelet and leukocyte antigens, siliconized glassware or a suitable plastic should be used.

The blood is centrifuged for 10 min at 180 g. The sediment containing erythrocytes and leukocytes is used for the preparation of the leukocyte antigen (see Sec. 7).

The plasma containing the platelets is decanted and spun down for 30 min at 1,600 g. The platelet sediment is suspended and washed three times with the platelet-washing solution (Na2 EDTA, 0.3-percent in saline). Each centrifugation is done at 1,600 g for 15 min. After the last washing, the platelets are resuspended in the washing solution and counted. Aliquots containing 2 x 10^9 platelets are centrifuged again. Each aliquot will be used for testing one serum. The packed sediment can be used either fresh or lyophilized. In the latter case, the dried antigen must be rehydrated with the appropriate amount of distilled water 16 to 20 hr before the test and kept at 4°C during this time. During sensitization by the serum to be tested, 0.05 ml of the 5-percent Na2 EDTA solution must be added in order to prevent clumping during this stage. After sensitization, the platelets are washed six times with the 0.3-percent Na2 EDTA solution. Each centrifugation is for 10 min at 1,600 g.

The further stages of A.H.G. consumption and titration are performed as described above.

7. Leukocyte Antigen

The blood is drawn into the 5-percent Na2 EDTA solution as described above, and then centrifuged at 180 g for 10 min. The plasma containing platelets is used for the preparation of the platelet antigen (see Sec. 6). The sediment containing the erythrocytes and the leukocytes is used for the preparation of the leukocyte antigen. One volume of sediment is suspended in two volumes of saline. Then one volume of a macromolecular substance is added in order to induce erythrocyte sedimentation. Any one of the following solutions can be used:

(1) polyvinylpyrrolidone, 3.5-percent in saline;

(2) dextran (molecular weight: 160,000), 5-percent in saline; and

(3) acacia solution prepared as follows: 10 g of white acacia powder and 1 g of anhydrous sodium phosphate (Na_2HPO_4) are put in a mortar, and 90 ml of distilled water are slowly added at the same time as the mixture is ground up into a homogeneous solution. The latter is autoclaved for 10 min at 116°C at a pressure of 1 kg. The container is kept at 4°C until a precipitate of colloidal calcium phosphate has sedimented out (approximately 24 hr). The supernatant, which is the acacia solution, is decanted and kept at 4°C. It must not be used for more than a week after preparation.

The mixture is thoroughly shaken and poured into 140 x 16 mm tubes. The tubes are inclined at 45° and the sedimentation of RBC takes place during 20 min at room temperature. The tubes are then placed in a vertical position and the sedimentation goes on for 10 min more.

The supernatant, which constitutes the leukocyte suspension, is decanted. A first centrifugation is carried out for 10 min at 180 g. The sediment is washed three times in 1.2-percent NaCl solution (10 min, 180 g).

After the last washing, the leukocytes are suspended in 1.2-percent NaCl solution and counted, and aliquots containing 15 x 10^7 leukocytes are centrifuged again. Each aliquot will be used for testing one serum. The packed sediment can be used either fresh or lyophilized. In the latter case, the dried antigen must be rehydrated with the appropriate amount of distilled water 16 to 20 hr before the test and kept at 4°C during this time.

The sensitization by the serum to be tested is done as described in Sec. 3. After sensitization, the leukocytes are washed six times in the 1.2-percent NaCl solution (10 min, 180 g). The further stages of A.H.G. consumption and titration are performed as described above.

8. Skin-Cell Antigen

A full-thickness piece of skin is surgically removed and the fat tissue adhering to the dermal layer is discarded. The skin is then frozen at −60°C and lyophilized. It is then cut into small pieces and ground in a mortar after addition of twice its weight of quartz. Sixteen to 20 hr before use, this

powder is rehydrated with a known volume of saline. Aliquots containing 2 mg of skin and 4 mg of quartz are prepared. Each aliquot will be used for testing one serum. The sensitization by the serum to be tested is done as previously described. The sensitized antigen is then washed six times in saline (10 min, 180 g). The further stages of A.H.G. consumption and titration are performed as described above.

Complement-Fixation Test for Antibody to Leukocytes

Henry E. Wilson

Fresh leukocyte suspensions may be employed each day, but previously prepared antigen may be kept frozen for considerable periods. Approximately 15 ml of blood are collected in a tube containing 0.5 ml of 5-percent disodium versenate and 1 ml of 6-percent dextran in physiological saline. Siliconized glassware is used throughout. The blood is placed in three 15 x 200 mm test tubes and allowed to settle for 1 to 2 hr at room temperature, after which the plasma suspension of platelets and leukocytes is removed with a Pasteur pipette. This suspension is placed in a 12-ml centrifuge tube and centrifuged at 750 rpm for 15 min. The supernate, consisting of plasma and platelets, is removed and the original volume is re-established by the addition of physiological saline. The leukocytes are twice washed by repeating the above procedure and are then resuspended in physiological saline in a concentration approximating 50,000 WBC/mm^3. All test sera are inactivated at 56°C for 30 min immediately before the test.

The leukocytes are fragmented in an ultrasonic vibrator for 2½ min at 400 kc/sec. If the ultrasonic vibrator is not available, fragmentation may be achieved by treating in a Virtis Blender, using a macro attachment with the setting at 40 for 10 min. Following fragmentation, the suspension is centrifuged in the cold at 2500 rpm for 25 min and the supernate used as the test antigen.

Complement and sensitizer must be individually titrated prior to the test. Rabbit anti-sheep serum is serially diluted in 0.25-ml quantities from 1:1,000 through 1:9,000 to determine the dilution which gives optimal lysis of 0.25 ml of 1-percent sheep red blood cells in 1-percent Mg saline in the presence of 0.1 ml of complement containing two *exact* units. This quantity is defined as one unit of sensitizer. By halving the dilution of sensitizer designated as one unit, one arrives at a quantity of sensitizer equivalent to two units in 0.25 ml. This dilution of sensitizer is then incubated in a waterbath at 37°C for 30 min in the presence of decreasing quantities of 1:30 complement (0.5 ml; 0.4 ml; 0.3 ml; 0.25 ml; 0.20 ml; 0.15 ml; 0.10 ml; 0.075 ml; and 0.050 ml) and 0.25 ml of sheep red blood cells suspended in 1-percent Mg saline. The highest dilution of complement which gives optimal lysis under these conditions is one *exact* unit. For use in the test, the complement dilution is adjusted to give two *exact* units per 0.1 ml.

In the routine procedure, two dilutions of serum, 1:1 and 1:4, are used. For the lower dilution, 0.2 ml of serum, and for the higher dilution, 0.05 ml of serum is pipetted into a 10 x 75 mm culture tube. To each tube is added 0.25 ml of antigen, 0.1 ml of guinea pig serum containing two *exact* units of complement, and sufficient Mg saline (1-percent $MgSO_4$ in 0.85-percent NaCl) to attain a volume of 1.0 ml. The mixture is shaken and incubated overnight at 4°C. Then 0.25 ml of a 1-percent suspension of sheep red blood cells in 1-percent Mg saline and 0.25 ml of rabbit anti-sheep serum containing two units of specific sensitizer are added to each tube. The final total volume in each tube is 1.5 ml. The mixture is now shaken and incubated in the waterbath at 37°C and read at 15 min and 30 min for fixation of complement. Antigen, serum, and hemolytic system controls are included in the series.

Reference

Johnson, H. M., H. E. Wilson, and M. C. Dodd. *In* Proc. Ninth Congress International Society Haematology, Mexico, 1962. Karger: Basel. (in press)

Complement-Fixation Test for Antibody to Platelets

Method described by: Wilson, Henry E., M.D.; Johnson, Howard M., Ph.D.; and Dodd, Matthew C., Ph.D: American Society of Hematology, Montreal, November 1960.

Fresh blood is obtained from healthy donors by venipuncture using a siliconized syringe which has been rinsed with 1-percent disodium versenate. After piercing the vein, the tourniquet is removed. Usually, 15 ml of blood are drawn. Whatever amount is drawn is added to a solution of 1-percent disodium versenate in a ratio of 13:2. The sample is centrifuged at 1,000 rpm for 15 min at 4°C and the platelet-rich plasma is drawn off and centrifuged at 3,000 rpm for 30 min at 4°C. The platelets are washed twice following the above procedure and finally the supernate is pipetted off and the platelets resuspended in physiological saline in a concentration approximating 400,000 platelets/mm³. No special care is necessary to maintain the integrity of these platelets thereafter for the complement-fixation technique. Frozen, stored platelets are as satisfactory as fresh platelets when used as antigen in this procedure.

All test sera are inactivated at 56°C for 30 min immediately before the test.

Complement and sensitizer must be individually titrated prior to the test, as described in the Complement-Fixation Test for Antibody to Leukocytes.

In routine procedure, two dilutions of serum, 1:1 and 1:4, are used. For the lower dilution, 0.2 ml of serum, and for the higher dilution, 0.05 ml of serum are pipetted into a 10 x 75 mm culture tube. To each tube is added 0.25 ml of antigen, 0.1 ml of guinea pig serum containing two *exact* units of complement, and sufficient Mg saline (1-percent $MgSO_4$ in 0.85-percent NaCl) to attain a volume of 1.0 ml. The mixture is shaken and incubated overnight at 4°C, after which 0.25 ml of a 1-percent suspension of sheep red blood cells in 1-percent Mg saline and 0.25 ml of rabbit antisheep serum containing two units of specific sensitizer are added to each tube. The final volume in each tube is 1.5 ml. The mixture is now shaken and incubated in the waterbath at 37°C and read at 15 min and 30 min for fixation of complement. Antigen, serum, and hemolytic system controls are included in the series.

Microdroplet Assay of Human Blood Lymphotoxins

P. I. Terasaki

To be practicable on a large scale, human leukocyte typing methods must be (1) reproducible; (2) simple, so that they can be used with numerous antisera and cells; (3) sensitive, so that valuable human antisera, which can almost never be obtained in large volumes, can be used in minute portions for each test; and (4) quantitative, for the accurate measurement of a reaction.

The microdroplet lymphocyte cytotoxicity assay fulfills the first three requirements, and can be made to be at least semiquantitative.

The test itself consists of reacting 0.003 ml to 0.00001 ml of antiserum dilution, 0.004 ml of absorbed rabbit C′, and 0.001 ml of a cell suspension containing 2,000 lymphocytes. These small volumes were added to oil chambers as in the de Fonbrune micromanipulation methods. Use of a dish-type chamber with a coverglass bottom and an inverted microscope facilitated the setting up and reading of the tests. Evaluation of cytotoxicity was made by morphologic appearance under phase-contrast microscopy.

The extreme sensitivity of the test (11 antisera were 50-percent cytotoxic at a dilution of 0.00001 ml) has made possible the uncovering of cytotoxins in the serum of half of 58 multigravid women tested. The simplicity of the test permitted titration of 50 different antisera with lymphocytes of as many as eight individuals on one day. Further details of the test have been submitted for publication elsewhere.

Normal Lymphocyte Transfer (NLT) Test in Humans

Charles B. Carpenter, Richard J. Glassock, and John P. Merrill

1. Preparation of Lymphocyte Suspensions

A modification of the original methods of Brent and Medawar (1963) was used.

Venous blood was withdrawn in nonsiliconized syringes and immediately transferred to glass jars (60 x 25 mm)* containing six to eight glass beads, each 3 mm in diameter. These screw-cap jars containing the glass beads were prepared in advance by dry-heat sterilization. The amount of venous blood withdrawn depended upon the volume needed for testing. Fifty milliliters of venous blood yielded on the average 0.4 ml of lymphocyte suspension containing approximately 3.8 x 10^6 lymphocytes per 0.1 ml. Immediately after addition to the jars the blood was agitated vigorously for not less than 10 min to ensure complete defibrination.

The defibrinated blood was then aspirated through an 18 gauge disposable needle, containing a small pledget of glass wool in the hub, into a syringe containing an equal volume of 6-percent dextran (average molecular weight: 188,000) in normal saline. The resultant mixture was transferred into 20-ml, capped, glass test tubes and allowed to sediment at 37°C for approximately 30 to 45 min. The supernate was then carefully aspirated with a 4- to 5-in., 18 gauge needle, transferred into a 50-ml graduated, screw-cap centrifuge tube, and centrifuged at room temperature for 10 min at 1500 rpm in an International PR-2 centrifuge (radius: 15 cm).

The supernatant serum-dextran-saline mixture was carefully aspirated; a small portion was reserved for later use as a control and the remainder discarded. The lymphocyte-rich sediment was resuspended in approximately 0.5 to 1.0 ml of supernate and allowed to resediment for approximately 30 min at 37°C in a waterbath in order to allow for further separation of erythrocytes. The final lymphocyte-rich supernate was aspirated *carefully* into disposable tuberculin syringes. The remaining sediment was tested for sterility by routine bacteriological methods. Aseptic precautions and sterile glassware were used throughout the preparative procedure.

The cellular content of the resultant suspension was determined by dilution in white blood cell pipettes, using 3-percent acetic acid as diluent and counting with phase microscopy in a hemocytometer chamber. Cover-glass smears were made of the final suspension, stained with Wright's Stain, and the percentage of lymphocytes was enumerated. Yield was expressed in numbers of lymphocytes per 0.1 ml of final suspension. Cell viability was tested by the trypan blue dye-exclusion method. Lymphocytes were rendered nonviable by four freeze-thaw cycles in a dry-ice—acetone bath. Final suspensions containing greater than 6 x 10^6 lymphocytes per 0.1 ml were diluted with appropriate amounts of serum-dextran-saline supernate. Final lymphocyte yield ranged from 1.1 to 5.3 x 10^6 lymphocytes per 0.1 ml. Fifty milliliters of whole venous blood yielded on the average 0.4 ml of final suspension with a range of 0.2 to 0.8 ml. Lymphocytes, most of which were small, comprised an average of 89 percent of the total cell count, suspensions with cell viability of less than 95 percent were discarded. Lymphocyte-to-red cell ratio in the final suspension was approximately 2:1.

2. Skin Testing and Reading

Lymphocyte suspensions freshly prepared from donors were injected intradermally into test recipients in 0.1-ml volumes using disposable tuberculin syringes and 25 gauge needles. Dextran hypersensitivity, history of hepatitis, or recent viral illness excluded lymphocyte donors and recipients.

Injections were made on the volar surface of one forearm and control injections of supernatant plasma-dextran-saline and in some cases freeze-thaw suspensions were made in a similar fashion on the other forearm.

* Wheaton Glass and Plastic Manufacturers, Millville, New Jersey.

Great care was taken to avoid direct intralymphatic injection of the suspension. Skin reactions were observed at 24 and 48 hr and thereafter as indicated. In 50 percent or more of the injections second ("delayed") responses occurred approximately 8 days after the initial reactions. Measurements of erythema and induration were made by at least two independent observers and recorded as maximal diameters in millimeters. The reactions were also recorded by color photographs and, in some cases, tissue-paper tracings for permanent records. Excisional biopsies were taken from selected cases at appropriate intervals. Lymphocyte suspensions, when yields permitted, were injected into their respective donors as a further (autologous) control.

In instances of single-donor—multiple-recipient lymphocyte panels, the skin reaction to viable lymphocytes was given a rank order according to the degree of erythema and induration at each observation. Whenever possible, delayed, control, and nonviable lymphocyte skin reactions were observed and recorded.

Reference

Brent, L., and P. B. Medawar. Tissue transplantation: a new approach to the "typing" problem. Brit. Med. J. 5352: 269–272, 1963.

Preparation of Viable Human Lymphocytes

J. G. Gray and P. S. Russell

In the first experiments, platelets and some of the polymorphonuclear leukocytes were removed by defibrination. Subsequently, carbonyl iron powder was employed to remove phagocytic cells.

1. Defibrination Method

Using sterile precautions throughout, 50 ml of venous blood are defibrinated by gently swirling in an Erlenmeyer flask containing three glass beads for each 10 ml of blood. After 25 ml of defibrinated blood were thoroughly mixed with 10 ml of 3.5-percent polyvinylpyrrolidone (PVP, molecular weight: about 25,000), the erythrocytes were allowed to sediment for 30 min at room temperature. Defibrination removed the platelets and many of the granulocytes, and thus eliminated some of the nonimmunological causes of skin reactions. The PVP was apparently nontoxic; among the various media tried, this has proved quite suitable for separating erythrocytes from leukocytes.

The supernatant was removed from the sedimented red cells, and centrifuged in conical tubes for 5 min at 1500 rpm. The supernatant was again removed, and a 0.5 ml portion of it was used to resuspend the deposited cells. At this stage, there was considerable red-cell contamination, and some granulocytes were still present. The concentrated cell suspension was then centrifuged at 800 rpm in tubes measuring 11 x 0.8 cm for 5 min. This deposited the red cells at the bottom, with the granulocytes adjacent, and left the lymphocytes in the supernatant.

This technique has produced a lymphocyte suspension containing an average of 30,000 cells/mm³. Lymphocytes comprised 95 to 100 percent of the preparation, 99 percent being viable by a trypan blue exclusion test. Erythrocyte contamination amounted to 20,000 cells/mm³.

2. Iron Powder Method

Fifty milliliters of venous blood are withdrawn into a heparinized* syringe. The blood is then mixed with 3-percent gelatin,** 3 ml of gelatin to 10 ml of blood.

After sedimenting for 30 min at 37°C, the supernatant is removed and to it is added carbonyl iron powder,*** 100 mg per 10 ml plasma. The plasma containing the iron powder is then gently agitated on a Yankee Rotator for 30 min at 37°C. This suspension is then centrifuged at 200 rpm for 5 min in an International Clinical centrifuge to deposit the phagocytic cells. The supernatant from this spinning contains the lymphocytes which are deposited by centrifugation at 1500 rpm for 5 min. The cells are then resuspended in 0.5 ml of platelet-free plasma and allowed to settle for 30 min at room temperature. The supernatant from this final preparation contains cells of which 95 to 100 percent are lymphocytes. Cell viability was between 90 and 100 percent.

* Panheprin. Abbot Laboratories, North Chicago, Illinois, U.S.A.
** Plasmagel. Laboratoire Roger Bellon, 189, avenue de Roule, Neuilly, Seine, France.
*** Carbonyl Iron Powder Type S.F. Antara Chemicals, 435 Hudson Street, Hudson, New York, U.S.A.

Method for Studying Lymphocyte Interaction and Other Immunologic and Cytogenetic Studies of Human Lymphocytes

Kurt Hirschhorn

1. Standard Method

(1) Thoroughly wet syringe with heparin (1:5000, free of phenol or cresol).

(2) Draw 20 to 30 ml of venous blood with fresh needle, and mix in syringe.

(3) Transfer to 16 x 100 mm test tubes with screw caps (nontoxic rubber liners).

(4) Place tubes at 45° angle at 37°C until erythrocyte sedimentation has taken place (½ to 1 hr).

(5) Pipette off WBC-plasma and mix with equal volume of Eagle's minimal essential medium modified for suspension culture (MEM-S), containing 100 units of penicillin and 100 μg streptomycin per ml.

(6) Put 10-ml aliquots into three oz. prescription bottles with nontoxic rubber-lined screw caps, and place on flat side at 37°C for 1 to 2 hr to remove the polys.

(7) Transfer to centrifuge tubes by gently pouring supernate without agitation, and spin at 800 rpm in clinical centrifuge for 10 min.

(8) Discard supernate, suspend cells in 10 ml of MEM-S, and centrifuge at 800 rpm for 5 min.

(9) Discard supernate and suspend cells in 5 ml medium (MEM-S with 20-percent fetal calf serum and 1 percent of a 200 mM solution of L-glutamine freshly added (glutamine should be kept frozen), mixing all cells from the sample.

(10) Using a WBC counting pipette, make 1:20 dilution with WBC counting fluid (0.1 N acetic acid with 1 to 2 drops of methylene blue per 100 ml) and count cells in blood-counting chamber (count only mononuclear cells).

(11) Make final dilution with medium to concentration of 750,000 mononuclear cells/ml.

(12) Set up cultures in 16 x 100 test tubes with non-toxic rubber-lined screw caps as follows: 4 ml blank from each individual, 4 ml culture with 0.1 ml phytohemagglutinin (PHA-M; if PHA-P is used, only 1/10 the amount is required) from each individual, 4 ml equal mixture (2:2) from each pair of individuals.

(13) Keep cultures at 37°C and harvest PHA culture at 3 days and others at 7 days. 2-4 hours before harvesting add 0.1 ml vincaleucoblastine (Velban, Eli Lilly) made up as a stock 0.5 μg/ml.

(14) To harvest, spin cultures at 800 rpm for 5 min, discard supernate, and suspend in 5 ml of 1-percent Na citrate.

(15) Immediately add 1 drop of fixative (three parts absolute alcohol to one part freshly prepared glacial acetic acid) and spin at 400 rpm for 5 min.

(16) Discard supernate and slowly, with constant mild agitation, suspend in 5 ml of fixative.

(17) Let stand 10 min, spin at 400 rpm for 5 min, discard supernate, and suspend in ½ ml of fixative.

(18) Prepare clean cover slips, and place 8 to 10 small drops of suspension on each cover slip with capillary pipette, blowing constantly to achieve even spreading.

(19) When dry, invert into a drop of 0.5-percent acetic orcein (0.5 g orcein in 45 ml glacial acetic acid, reflux ½ hr, add 55 ml distilled water while warm, reflux ½ hr, let stand 24 hrs., filter, and filter fresh before using) on a clean slide, suck out excess stain with filter paper, and seal with Kroenig's cement. If permanent slides are desired, treat dry cover-slip preparation in following sequence: 0.5-percent acetic orcein (30 min), 45-percent acetic acid (dip until free of excess stain), tertiary butyl alcohol (15 sec), tertiary butyl alcohol (15 sec), 1:1 tertiary

butyl alcohol:xylene (1 min), xylene (1 min), xylene (1 min), and invert wet into Permount (thinned with xylene) on clean slide.

(20) Examine slide with phase microscope (if bright field illumination is desired, stain with 1-percent acetic orcein, prepared as above, but with 1 gm. orcein) for percentage of small cells, large cells, and mitoses, counting at least 1,000 cells from randomly chosen fields. A cell is called large either by size alone or by reticular staining of nucleus.

2. Modifications for Special Purposes

a. *Studies in sensitized individuals.*

(1) Repeat step (8) three times.

(2) For cell-cell interaction, harvest mixtures and blanks at 5 days.

(3) For studies with specific antigens, add these at step (12) and harvest at 5 days with blanks.

b. *Chromosome studies.*

(1) Omit step (8) unless these are to be done in cell-cell mixtures.

(2) In step (12) use only PHA.

(3) In step (15) wait 7 min before adding 1 drop fixative, and spin at 200-400 rpm for 5 min.

(4) For chromosome studies in cell-cell mixtures, same modifications at step (15).

c. *Microcultures for chromosome or antigen studies.*

(1) Prepare 5-ml aliquots of medium containing 0.25 ml heparin.

(2) From free-flowing puncture of cleansed skin (70% EtOH, then acetone or ether and let dry), aspirate blood with sterile Pasteur pipette, place 2 drops of blood in each tube and mix well.

(3) Add 0.1 ml of PHA or antigen and keep at 37°C for 3 days (PHA) or 5 days (antigen).

(4) For chromosome studies, treat as in Sec. 2.b, except that 0.7-percent Na citrate is used for 15 min at 37°C, and that fixation is carried out for 20 min at 4°C.

d. *For fluorescence studies.*

(1) Use 1 x 10^6 cells/ml of culture.

(2) Substitute agamma calf serum for fetal calf serum in medium.

(3) Repeat step (8) three times.

(4) Harvest cultures at 24 or 48 hrs. (without Velban), and proceed as follows: spin at 400 rpm for 5 min, discard supernate, suspend in 5 ml MEM-S, spin at 400 rpm for 5 min, discard supernate, and repeat this washing two more times; suspend by gentle pipetting in 0.5 ml MEM-S containing 1 drop of the desired fluorescein-conjugated anti-human gamma globulin (e.g., anti-7S, anti-gamma, A, anti-gamma, M), incubate at 37°C for 45 min, agitating every 15 min; spin at 400 rpm for 5 min; and repeat above washing procedure three times, using phosphase-buffered saline (PBS); suspend in 0.25 ml PBS, place several micro-droplets of suspension on cover slips, and air-dry; invert cover slip into nonfluorescent oil on a slide, and seal; examine with ultraviolet light and phase illumination to count the number of fluorescing cells.

MEM-S and sera obtained from Grand Island Biologicals, Grand Island, N. Y.

Techniques: Mixed Leukocyte Cultures

Barbara Bain and L. Lowenstein

1. Preparation of Cultures

(1) Blood from each of two subjects is drawn into a heparinized* syringe and placed in disposable plastic culture tubes.

(2) The tubes are centrifuged for 5 min at 1500 rpm (500 g).

(3) The supernatant plasma, buffy-coat layer, and the upper portion of the red cells, are transferred to another tube and gently resuspended with a Pasteur pipette.

(4) The tube is kept at an angle of about 30° to vertical, at 37°C, for 30 to 60 min, to allow the red cells to settle. It is then centrifuged for 5 min at 300 rpm (25 g).

(5) The supernatant plasma, containing the leukocytes, is removed to another tube with a Pasteur pipette. The leukocytes are counted.

(6) The supernatant is diluted with 199 medium, so that the calculated cell count is about 1000/mm³. The final plasma dilution is from 4 to 10 times, cell-free plasma being added if necessary.

(7) Aliquots of the diluted cell suspension are distributed into 17 x 100 mm disposable plastic culture tubes:

(a) unmixed controls: 4 ml from each subject alone; and

(b) mixtures: 2 ml from each of the two subjects, together in the same tube, so that the total volume is 4 ml.

(8) The culture tubes are placed upright in an incubator at 37°C, and are left undisturbed for 5 days. At the end of this time, H^3-thymidine uptake is measured.

2. Measurement of H^3-Thymidine Uptake

(1) After 5 days of incubation at 37°C, the cells are resuspended by gentle shaking. H^3-thymidine, 0.1 ml of 40-μc/ml stock solution (spec. act. about 35 mc/mg) is added to each tube to give a final concentration of 1 μc/ml.

(2) One hour later, the tubes are centrifuged and supernatant fluid is discarded. Two milliliters of physiological saline are added to each tube, and the cells are resuspended.

(3) Step (2) is repeated twice more, so that the cells are washed three times with saline.

(4) After the final centrifugation, as much fluid as possible is removed, and 0.5 ml of Hydroxide of Hyamine (1 M in methanol**) is added to each tube.

(5) The tubes are kept at room temperature in the dark for 2 days. The contents are then transferred to screw-capped counting vials with a Pasteur pipette. The residue from each tube is washed over into the vial with two 0.3-ml washings of absolute ethyl alcohol. The caps are screwed on tightly.

(6) The counting vials are placed in a water-bath, and the temperature control is turned to 75°C. After 1½ hr, the heat is turned off. When the vials are cool enough to handle, they are dried with a cloth and allowed to stand for at least 30 min, so that the moisture under the rim of the cap evaporates.

(7) Nineteen milliliters of a solution containing 0.3-percent PPO** and 0.01-percent POPOP** in toluene are added to each vial. The vials are now ready for counting in a liquid scintillation counter.***

We usually prepare three cultures from each unmixed control, and four cultures from the mixture. The H^3-thymidine uptake is measured in duplicate for the controls and in triplicate for the mixture. The remaining cultures are used to make smears, which are stained with Jenner-Giemsa stain.

3. Result

When the two subjects are unrelated, H^3-thymidine uptake in the leukocyte mixture is greatly increased over that of the unmixed controls, usually by a factor of 10 or more.

* "Liquaemin," Organon.
** Packard Instrument Co.
*** E.g., Packard Tri-Carb, Model 3002.

Mixed Agglutination with Cell-Culture Monolayers and Homotransplantation Antibody

F. Milgrom, J. Abeyounis, and K. Kano

1. Introduction

Mixed agglutination with cell cultures was introduced by Högman (1959) and Espmark and Fagraeus (1962). Using potent heteroimmune sera, it was demonstrated in previous studies (Milgrom *et al.*, 1964) that predominant antigens on living mammalian cell cultures are saline nonextractable species-specific antigens. In following this line of investigation, it appeared interesting to find out whether transplantation sera are capable of detecting isoantigens on proper cell cultures (Abeyounis, Milgrom, and Witebsky, 1964).

2. Procedure of Mixed Agglutination with Mouse-Cell Cultures and Mouse-Transplantation Sera

Most of the procedures employed in mixed agglutination have been discussed in previous publications (Milgrom *et al.*, 1964; Barron *et al.*, 1963; Kano and Milgrom, 1964; Abeyounis, Milgrom, and Witebsky, 1964).

a. *Media.*

(1) Phosphate-buffered saline (PBS).

(2) Eagle's basal medium in Hank's balanced salt solution (BSS), supplemented with 10-percent fetal calf serum.

(3) 0.5-percent Lactalbumin hydrolysate in Hank's BSS (LH medium).

b. *Sera.*

(1) C57BL anti-C3H serum obtained from female C57BL recipients grafted with skin from female C3H donors.

(2) Mouse anti-human erythrocyte serum prepared by i.p. injections of mice with a pool of human red blood cells.

(3) Rabbit antiserum against mouse serum prepared by immunizing rabbits with whole serum precipitated by potassium alum.

c. *Cell cultures.* Strain 929 "L" cell fibroblasts originally derived from a C3H mouse are used. The cells are grown in Eagle's basal medium in Hank's BSS supplemented with 10-percent fetal calf serum. The cell cultures attain a complete monolayer in 6 to 8 days, at which time they are used.

d. *Indicator system.* Equal volumes of a 4-percent suspension of human O Rh+ erythrocytes and of a 1:600 dilution of mouse anti-human erythrocyte serum are mixed and incubated at room temperature for 1 hr. The sensitized red blood cells are washed three times with PBS and resuspended to a concentration of 2 percent. Equal volumes of the 2-percent suspension of sensitized erythrocytes and the rabbit anti-mouse serum at 1:20 dilution are mixed and incubated at room temperature for 30 min. The agglutinated red blood cells are washed three times in PBS and are resuspended in the LH medium to a concentration of 0.25 percent. The agglutinated cells may be safely stored after the final washing for 4 to 6 hr at 4°C as a centrifuged button.

e. *Test.* The cell-culture monolayers are washed three times with LH medium and drained. Serial dilutions of the serum to be tested are made in LH medium and are added in 0.5-ml volumes to cell cultures.

The "L" cells are incubated with the antiserum for 2 to 3 hr at room temperature. The cell cultures are then washed five times with LH medium and drained. Five-tenths of a milliliter of the indicator system is added to each tube and the cell cultures are incubated at room temperature for 1 hr. The rack containing the culture tubes is shaken gently and it is allowed to stand upright for 1 to 2 min. The monolayers are examined under the microscope using low-power magnification.

3. Comment

In following the described procedure, positive results were obtained with sera of practically all tested C57BL mice grafted

with C3H skin. Sera usually were positive at the end of the 2nd week after transplantation and titers up to 1:10,000 were recorded (Abeyounis, Milgrom, and Witebsky, 1964). With primary cultures of mouse tissues, comparable results have been observed.

In addition to mouse transplantation sera, sera from homografted rats and rabbits were investigated, using primary cell cultures originating from the respective skin donor. Proper indicator systems were employed which consisted of erythrocytes sensitized by subagglutinating doses of antierythrocyte serum (of rat or rabbit origin) and agglutinated by the corresponding Coombs' reagent (rabbit anti-rat serum and goat anti-rabbit serum). Results were comparable with those obtained in the mouse system. From preliminary studies, it was concluded that the "human" indicator system involving tanned red blood cells coated by FII preparations does not give satisfactory results. Attempts are being made to elaborate a human indicator system using antibody-sensitized erythrocytes.

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Mixed Agglutination Technique for the Detection of Isoantigens on Human Kidney Cells

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The basic technique of mixed agglutination in tissue culture as described by Fagraeus and Espmark (1961) and Barron *et al.* (1963) is used. It has been adapted to demonstrate isoantigens on human kidney cells using human sera containing leukoagglutinins.

1. Preparation of Primary Human Kidney Cultures

Primary human kidney cultures are prepared from surgical specimens by a modification of the trypsinization procedure of Dulbecco and Vogt (1954). Cortical tissue from the kidneys is cut into small fragments of 0.5-cm diameter, washed twice in balanced salt solution and subjected to three cycles of treatment with Hank's solution containing ¼-percent trypsin. Trypsinization is performed by stirring the tissue fragments for 30-min periods at room temperature and decanting the supernatant at the end of each treatment. The cells from all three trypsinization treatments are pooled in 10 ml of mixture 199 containing 20 percent calf serum. The count is adjusted to 1 million cells per ml and the cells are transferred to 4-oz prescription bottles in a volume of 5 ml or into 16 x 125 mm screw-capped tubes in 1-ml quantities. The feeding fluid is replaced 24 hr later to remove debris and dead cells from the medium. Thereafter the cells are fed every 48 hr with the same medium used for isolation. Confluent sheets of cells are formed at the end of 4 to 6 days. The cultures are used for serologic studies at that time or passed to new tubes. The second passage almost always results in homogeneous cultures which are more suitable for experimental work than are the primary cultures. Transfer of cells to new culture vessels beyond this point uniformly failed.

2. Preparation of Indicator System

Human group O erythrocytes diluted to 4 percent in pH 7.2 phosphate buffered saline are mixed with an equal volume of 1:20,000 tannic acid. The mixture is incubated at room temperature for 30 min, and thrice washed with pH 7.2 buffered saline. The erythrocytes are diluted to a 2-percent concentration. The 2-percent tannic acid-treated cells are then mixed with an equal volume of human globulin (0.08-percent Cohn Fr. II) and incubated at room temperature for 30 min. The cells are then washed three times with buffered saline and resuspended to a 1-percent concentration. The tanned globulin-coated cells are then combined with an equal volume of 1:50 goat antiserum to whole human serum, incubated at room temperature for 30 min, washed three times, and resuspended to a 0.25-percent concentration in Hank's solution.

3. Test Procedure

The 16 x 125 mm tissue-culture tubes are rinsed three times with 2.0 ml of Hank's solution in order to remove the calf serum present in the medium. The last wash is then replaced by 1 ml of the antisera or normal sera diluted in Hank's solution. The tubes are then incubated at room temperature for 2 hr, and again thrice rinsed with 2.0-ml volumes of Hank's solution. The indicator system (1.0 ml) is then added and the tubes incubated for 1 hr at room temperature. After the incubation period, the tubes are gently agitated to resuspend the indicator cells that are not bound to the tissue cells and then inverted for reading, so that the tissue culture surface is at the top of the tube and the indicator fluid is at the bottom. A positive reaction occurs when antibody has fixed to the tissue-culture cells and has reacted with the goat antiserum-red cell indicator system. Multiple clumps adhere to the monolayer and cannot be detached with marked agitation of the indicator-cell fluid. A negative reaction is seen when all the indicator cells settle to the bottom of the tube opposite the monolayer. An occasional clump that adheres to the monolayer can be easily dislodged by gentle agitation.

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Addendum

The technique of mixed agglutination has also been used to detect human tissue isoantigens on continuous cell lines of human origin (KB, HeLa, H.Ep. No. 2, and FL amnion). The antisera used in these experiments were from multiparous women and immunized chimpanzees and were known to contain leucoagglutinins. The following procedure can also be used for preparation of the indicator system.

Human group O, D positive (Rh_o) cells are tested with a 1:500 dilution of a human incomplete anti-D (Rh_o) serum for 15 minutes at room temperature. The cells are then washed three times with 0.85% saline, resuspended to a 2% concentration and agglutinated by an equal volume of a 1:100 dilution of goat antiserum to human serum. The cells are then washed again three times with saline and resuspended to a 0.25% concentration in Hank's solution.

Since the anti-D indicator system is easier to prepare and gives a more stable cell suspension, it is now used as the routine indicator system. It was also found that controls completely void of non-specifically adhering red cells could be obtained by pouring off the indicator system after the 1 hour incubation period and replacing this with 2 ml. of media. The erythrocytes adhering to the tissue culture cells specifically sensitized with antibody were not affected by this treatment.

General Description of Method of Grafting and of Graft Observation of Human Subjects

Felix Rapaport

1. Method of Sensitization

Under local 1-percent procaine infiltration anesthesia, the donor site is incised with a circular trephine (11 mm in diameter) and a full-thickness skin graft is excised. The graft is placed on a segment of gauze saturated with normal saline, and the undersurface is freed of subcutaneous tissue. A graft-bed area identical in size is prepared in similar fashion in the recipient on the radial aspect of the volar surface of the forearm. After complete cessation of bleeding and capillary oozing in the recipient bed, the graft is inserted into the defect, and its edges are approximated to the recipient's skin with interrupted 5-0 nylon sutures. The area is covered with a layer of fine nylon mesh and a dry, sterile pressure dressing is applied. Alternate sutures are removed on the 3rd day, and the remaining sutures, on the 4th day.

2. Method of Observation and Criteria of Homograft Rejection

Two methods are used routinely to determine the onset of the rejection changes in skin grafts, as described below.

a. *Gross appearance of graft rejection.* The gross characteristics of rejection include graft cyanosis, hemorrhage, and edema, with an area of surrounding erythema and induration in the recipient skin. The ultimate fate of the rejected graft is a dry eschar which is sloughed as host epithelium infiltrates the subeschar surface. These changes are regularly preceded by the stereomicroscopic findings described in Sec. 2.b.

b. *Stereomicroscopic criteria of graft rejection.* The method of stereomicroscopic observation of graft surface capillaries devised by Taylor and Lehrfeld has been adapted to man throughout this series of experiments. Briefly, it consists of the examination of the graft surface capillary circulation through a film of mineral oil, by use of a Bausch and Lomb stereomicroscope under magnifications of 19.5 X, 45 X, and 90 X. This method permits the prediction of the ultimate macroscopic rejection of the skin graft by observation of the microscopic alterations which herald the rejection changes. These include:

(1) cessation of capillary blood flow, (2) intracapillary thrombosis, and (3) extravasation of blood into the graft.

All grafts are examined at daily intervals from the 3rd postoperative day until rejection. This permits early detection of the onset of graft rejection changes; it also serves to indicate the onset of blood flow in the capillaries of homografted skin, as a critical measure of satisfactory union between host and graft.

c. *Summary.* The criteria for graft rejection developed in the course of this study include: (1) development of erythema and induration around the graft; (2) cyanosis and edema of the graft; and (3) cessation of blood flow and capillary thrombosis in the graft vessels. The eventual development of an eschar and its sloughing are required to confirm the diagnosis of graft rejection in each case.

By means of these criteria, it has been determined that the average time of rejection of first-set grafts is 8 to 12 days, whereas a second graft from the same donor to the same recipient, if applied 2 to 3 weeks later, is rejected in accelerated fashion (4 to 6 days). Rechallenge of the recipient with a second-set graft within the 1st week after first-set rejection results in a white-graft reaction. The latter is characterized by complete lack of vascularization and gradual change of the graft into a tan-colored eschar.

Summary

D. Bernard Amos

Following the 1-day conference on tissue typing, a practical workshop was held at Duke University Medical Center at which many of the techniques and variations could be demonstrated by those most competent to perform the tests.

Actual bench demonstrations were performed as indicated in the program. Skin grafts were exchanged 2 days before the start of the meeting. Lymphocyte transfer tests involving the skin graft and other subjects were set up on the 1st day and inspected on each day of the meeting. Tissue cultures were brought by Drs. Milgrom, Bain, and Lowenstein, and other cultures were set up during the meeting by Dr. Hirschhorn.

The same samples of antisera, supplied by several different investigators, were used for the agglutination tests. Cells were obtained from a panel of eight group O donors and prepared under the supervision of the investigators concerned.

Numerous informal discussions took place during the 3 days of the meeting, and a formal discussion was held after the completion of the laboratory demonstrations. This session was conducted in sequence by Drs. van Rood and Amos and the whole conference was summarized by Dr. Ceppellini.

During the final discussion, results of the agglutination tests were compared. All the methods used were capable of distinguishing the negative control serum and all scored a number of positive results with each of the antisera. Some tests, especially that of Dr. Lalezari, gave extremely strong agglutination, but there were considerable differences between the incidence of positive results obtained with different methods when reactions against individual cells were compared. In this particular series of tests, there was close agreement between the results obtained by Drs. Payne and Dausset. These two techniques, using cells from defibrinated and EDTA-treated blood respectively, also gave a high frequency of positive reactions. On the basis of such a limited series of tests performed under unusual conditions, any attempt to draw conclusions would be unjustified, but there was a strange contrast between the strong agglutination and high titers observed in some tests and the higher incidence of positive reactions, but with loose clumping of the cells, obtained by other methods. It was obvious that more information is needed as to whether some techniques are detecting false positive reactions, whether some are missing true specific agglutination, and whether different types of tests are detecting different antibodies.

These results provoked much discussion, and it was generally agreed that no test presently available is ideal. Urgently required is a micro or semimicro method with both high sensitivity and specificity. Lacking this, it is essential to use more than one method, especially to confirm results obtained in another laboratory. In this case, when serum is exchanged the technique used in the original laboratory should also be used for checking the results. Wherever possible, and especially for purposes of phenotyping, a number of sera with the same apparent specificity should be used.

To aid in standardization of reagents, it was felt that two types of panel programs should be organized. One should be a large international panel to which any monospecific serum thought to define an antigen of a new system could be sent for checking against antigens already known. In addition, smaller panels should be established and typed with known sera, as these become available, at each of the other laboratories. This would prevent undue loss of time in rediscovering previously identified antigens. It is at present impossible to send cells for agglutination from one laboratory to another, but cells can be sent for absorption, or serum can be sent from one laboratory to another, tested against the local panel, absorbed, and retested, and finally samples of the absorbed serum sent back for confirmatory tests against the original panel. Dr. van Rood offered to organize a central reference panel in Leiden.

Dr. Terasaki performed cytotoxicity tests with several sera and, following suggestions

from several participants, I set up a modified agglutination test using a similar micro method but omitting complement and using cells prepared as for several of the conventional leukoagglutination tests. There was excellent agreement between duplicate tests set up blind within the main cytotoxicity test, and the test was impressive in giving clear-cut negative values with control sera and strong positive reactions with many of the test sera. The sera used in the agglutination tests were also tested for cytotoxic properties but, although several were cytotoxic, there was no close agreement between the two reactions. The micro agglutination test proved to be relatively insensitive at this first attempt. Agglutination was most clearly shown with cells prepared in EDTA.

The lymphocyte transfer tests served mainly as a demonstration of the methods. The experimental design was extremely complex, since (to avoid unnecessary risks to normal subjects for demonstration purposes) many of the subjects had been used for previous tests. All the recipients had been immunized at least once and two had been repeatedly immunized. As expected, there was considerable variation in reactivity both between subjects and between cells, but the weakest reactions were observed in the subjects who had been most strongly immunized. There were also differences between comparable tests performed with cells prepared in different ways.

The mixed agglutination tests both yielded interesting results. The titers obtained by Dr. Milgrom with the mixed agglutination test using L cells were considerably higher than titers obtained with the same serum when reacted against C3H red cells by Dr. Mark in the dextran system. The titers obtained by Dr. Metzgar with primary cultures of human kidney cells were generally similar to those obtained by leukoagglutination.

With respect to these newer tests, it was felt that the mixed agglutination methods were practicable and offered an excellent opportunity for testing for antigens not detectable by tests with leukocytes. The various lymphocyte tests were discussed with considerable interest. *If* the reactions are a direct reflection of the degree of antigenic difference between donor and host, then both will offer a means not only for direct matching of donor and recipient, but also for determining which of the antigens detected serologically are truly transplantation antigens.

In his summary, Dr. Ceppellini stressed the following points:

Much basic information is lacking with respect to the agglutination tests. Leukocytes are much more complex than erythrocytes and little is known about their cell membranes. There is too little information about the effect of variations in the physiological state of the white cell upon the process of agglutination. As yet, little is known about the antibodies. Some are certainly 7S gamma-globulin, but this is not necessarily true of all antibodies; and some intermediate classes, neither 7S nor 19S, might be important, just as they are for the ABO system. Cofactors may also play a role, particularly complement, as is suggested by the knowledge that specificity and sensitivity of the two main variants of the leukoagglutination test, i.e., using cells from defibrinated or EDTA-treated blood, are often quite different. It has, in fact, been shown that, although the addition of small amounts of fresh serum to the cell suspension appears to help agglutination, higher amounts can be inhibitory. The presence of inhibitors may also be inferred from other observations. Absorption of some sera by nonreactive cells may augment the reaction against otherwise weakly positive cells, while heating of certain sera also seems to accentuate agglutination. Prozones are common in some of the tests but rarely seen in other methods. A rheumatoid-like factor, possibly especially prevalent in polytransfused subjects, may reflect the presence of antiglobulins. The presence of a variety of antibodies in many of the sera can be inferred from the discrepancy between the cytotoxicity and agglutination tests.

The number of personal variants in running the agglutination tests, which have been shown by the participants in the workshop, is in itself evidence that an ideal methodology has not yet been found. Nonetheless, the test is working and its proof derives from the identification of an increasing number of antigen systems, where the fit of the genetic data gives confidence about the intrinsic consistency of the serological results. In this regard, the contributions of Dr. van Rood and his collaborators deserve special mention.

Of course, the cytotoxicity test of Dr. Terasaki and the mixed agglutination test on tissue cultures of Dr. Milgrom are also of the greatest interest and they must be studied in parallel with leukoagglutination.

These tests and any other methods for detecting polymorphism of antigenic structures in different tissues by serological reactions, represent "tissue typing," while other biological tests, *in vivo* (normal lymphocyte transfer) or *in vitro* (mixed lymphocyte culture), although possibly very important in practice as efficient methods for measuring degrees of histocompatibility, do not allow an identification of the discrete units upon which histocompatibility is founded. The most urgent problem, however, is to prove whether any of the antigens detected by serological methods has any relevance for the homotransplantation problem. It was suggested at a recent meeting of the New York Academy of Sciences that skin grafting is too exacting a test and, besides, is very laborious from the logistic point of view. But at present there seems to be no immediately practicable alternative, and each tissue antigen must be evaluated against transplantation experiments. In this respect the *in vitro* biological tests may become of the greatest interest. There are already good reasons for thinking that the "blast transformation" in mixed lymphocyte cultures may be a measure of the histocompatibility of the two individuals. If that is confirmed, this *in vitro* biological test can be substituted for skin grafting in the evaluation of tissue antigens. In any case the test is of great theoretical importance and corresponds to the *in vivo* test of Brent and Medawar. The latter appears to have the features of a graft-*vs.*-host reaction; the meaning of the test in man needs further evaluation.

Dr. Ceppellini concluded by pointing out the need for continued close cooperation. The number of investigators involved in tissue typing is still small and communication is relatively easy. Nevertheless, it is important to come to some decision over terminology and the identification of sera, and this, together with the establishment of a major international panel and collaboration in the setting up of smaller reference panels at a number of laboratories, is a matter of urgency.

Program of the Workshop

9 June 1964: Evening Session
- General Plans for the Workshop
  - Dr. Chester M. Zmijewski

10 June 1964: Morning Session
- General Presentation of Agglutination Methods
  - Dr. D. Bernard Amos
  - Dr. Rose M. Payne
  - Dr. Jon J. van Rood
  - Dr. Parviz Lalezari
  - Dr. Jean Dausset
  - Dr. Henry E. Wilson
- Setting Up and Reading Agglutination Tests
  - Dr. Jean Dausset
  - Dr. Parviz Lalezari
  - Dr. Rose M. Payne

10 June 1964: Afternoon Session
- First Examination of Skin Grafts
  - Dr. Felix T. Rapaport
- Setting Up and Reading Agglutination Tests
  - Dr. D. Bernard Amos
  - Dr. Jon J. van Rood
  - Dr. Henry E. Wilson and
  - Mr. Michael Van Winkle

11 June 1964: Morning Session
- General Presentation of Methods
  - Complement-Fixation
    - Dr. Henry E. Wilson
  - Complement
    - Dr. Henry J. Winn
  - Coomb's Consumption
    - Dr. Jacques Colombani
  - Mixed Agglutination
    - Dr. Felix Milgrom
  - Cytotoxicity
    - Dr. Paul I. Terasaki
- Demonstrations of Methodology
  - Dr. Henry E. Wilson and
  - Mr. Michael Van Winkle
  - Dr. Jacques and Mrs. M. Colombani
  - Dr. Felix Milgrom and
  - Mr. John Abeyounis
  - Dr. Richard Metzgar

11 June 1964: Afternoon Session
- Second Examination of Skin Grafts
  - Dr. Felix T. Rapaport
- Demonstrations of Methodology
  - Dr. Paul I. Terasaki
  - Dr. Felix Milgrom and
  - Mr. John Abeyounis

12 June 1964: Morning Session
- General Panel Discussion on Skin Grafting and *in vitro* and *in vivo* Lymphocyte Reactivity
  - Panel: Drs. Brent, Ceppellini, Glassock, Gray, Hirschhorn, Lowenstein and Rapaport
- Reading of Complement Fixation Tests
  - Dr. Henry E. Wilson and
  - Mr. Michael Van Winkle
- Interpretation of Skin Grafts
  - Dr. Felix T. Rapaport
- Demonstrations
  - Skin Grafting
    - Dr. Leslie Brent
  - Lymphocyte Cutaneous Tests
    - Dr. J. G. Gray
  - Normal Lymphocyte Transfer Test in Humans
    - Dr. Richard J. Glassock
  - Examination of *in vitro* Lymphocyte Reactivity
    - Dr. Louis Lowenstein and
    - Miss Barbara Bain
    - Dr. Kurt Hirschhorn

12 June 1964: Afternoon Session
- General Discussion of the Results of the Tests Demonstrated
  - Comparison of Methods
    - Dr. D. Bernard Amos
  - Closing Remarks
    - Dr. Ruggero Ceppellini

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