Symposium on the Laboratory Propagation and Detection of the Agent of Hepatitis (1954)

Pages
122

Size
6 x 9

ISBN
0309360439

Panel on the Sterilization of Blood and Plasma; National Research Council; Commission on Virus and Rickettsial Diseases, Armed Forces Epidemiological Board

The cost of this Symposium was defrayed by funds provided by the Research and Development Division, Office of the Surgeon General, Department of the Army.

7430

Symposium On The Laboratory Propagation and Detection of The Agent of Hepatitis

Held Under the Joint Sponsorship of

THE PANEL ON THE STERILIZATION OF BLOOD AND PLASMA

of the

NATIONAL ACADEMY OF SCIENCES—NATIONAL RESEARCH COUNCIL

and

THE COMMISSION ON VIRUS AND RICKETTSIAL DISEASES

of

THE ARMED FORCES EPIDEMIOLOGICAL BOARD

Bellevue Hospital, New York City

31 March 1954

Dr. John R. Paul, *Chairman*

Publication 322

NATIONAL ACADEMY OF SCIENCES — NATIONAL RESEARCH COUNCIL

Washington, D. C.

1954

$1.00

Participants and Guests

SYMPOSIUM ON THE LABORATORY PROPAGATION AND DETECTION OF THE AGENT OF HEPATITIS

Dr. John R. Paul, *Chairman*
Yale University
School of Medicine

Dr. Frederik B. Bang
Johns Hopkins Hospital

Dr. Stanhope Bayne-Jones
Research and Development Division
Office of the Surgeon General
Department of the Army

Cdr. S. A. Britten, MC USN
Bureau of Medicine and Surgery
Department of the Navy

Dr. Gilbert Dalldorf
New York State Department of Health

Dr. John F. Enders
Harvard Medical School

Dr. Alfred S. Evans
University of Wisconsin
School of Medicine

Capt. Arthur C. Fox, USAF (MC)
National Research Council

Lt. Col. William S. Gouchenour, Jr., VC USA
Office of the Surgeon General

Dr. W. Paul Havens, Jr.
Jefferson Medical School
Philadelphia, Pennsylvania

Dr. Gertrude Henle
Children's Hospital
Philadelphia, Pennsylvania

Dr. Werner Henle
University of Pennsylvania
School of Medicine

Dr. Fred O. MacCallum
Central Public Health Laboratory
London, England

Dr. Colin B. MacLeod
New York University
College of Medicine

Dr. George S. Mirick
Baltimore City Hospitals

Dr. Alice E. Moore
Sloan-Kettering Institute

Dr. J. Anthony Morris
Army Medical Service Graduate School

Dr. Roderick R. Murray
National Institutes of Health

Col. Adam J. Rapalski, MC USA
Administrator, AFEB

Dr. A. B. Sabin
University of Cincinnati
School of Medicine

Maj. Gerald J. Schipper, USAF (MC)
Office of the Surgeon General

Dr. Richard E. Shope
Rockefeller Institute for Medical Research

Dr. Margaret H. Sloan
National Research Council

Dr. Joseph E. Smadel
Army Medical Service Graduate School

Dr. John C. Snyder
Harvard School of Public Health

Dr. Joseph R. Stokes, Jr.
University of Pennsylvania
School of Medicine

Dr. Jerome T. Syverton
University of Minnesota
School of Medicine

Dr. William S. Tillett
New York University
College of Medicine

Dr. Gerard M. Turino
First Medical Division
Bellevue Hospital

Dr. Robert Ward
New York University
College of Medicine

CONTENTS

SYMPOSIUM ON THE LABORATORY PROPAGATION AND DETECTION OF THE AGENT OF HEPATITIS

I. Introduction and Purpose of the Symposium

Dr. Paul, Chairman, opened the meeting by welcoming the participants in this Symposium, which was being sponsored jointly by the Panel on the Sterilization of Blood and Plasma of the National Research Council and by the Commission on Virus and Rickettsial Diseases of the Armed Forces Epidemiological Board. On behalf of the Commission he wished to thank the National Research Council for their part in initiating and co-sponsoring the meeting. He added that he was particularly pleased to see that Dr. MacCallum, who had been among the first to study viral hepatitis intensively, had been able to make the journey from England in order to be present.

Dr. Paul then explained that the Conference had been planned to collect in one review most of the work which had been done and much of what had been left undone in laboratory efforts to propagate and detect the virus of human hepatitis. It was planned to review and document the many negative and positive experiments of the past and, by critical discussion, to determine what studies should be carried out in the future.

Dr. Paul then called on Dr. Tillett to describe further the purposes for which the Symposium was organized.

Dr. Tillett stated that for the past three years the National Research Council Panel on the Sterilization of Blood and Plasma, of which he had been chairman, had been charged with the difficult task of studying proposed methods of eliminating the hepatitis virus from blood and plasma. The ultimate test of each new method of sterilization required the inoculation into human volunteers of material of established, carefully titrated infectivity after it had been treated by the new method. Dr. Roderick Murray, of the National Institutes of Health, had for the most part, conducted this very difficult study. Since the need for using human volunteers was one of the greatest drawbacks in the search for a sterilizing method, the Panel members had considered for some time the ways in which they could stimulate efforts to transmit human hepatitis to animals, or better, to cultivate the virus. Many varying reports had been received of successful and unsuccessful attempts at transmission of the human disease to a large number of animal species, and of successful propagation of the agent in hens' eggs, tissue cultures, or other laboratory media. Many of these attempts had been poorly documented, and the nature, amounts, and routes of administration of the infecting materials used were often not specified. This Symposium was planned in order to review the past work critically and to delineate what efforts were now under way or should be initiated. Dr. MacLeod, of the Armed Forces Epidemiological Board, and Dr. Cannan, of the Division of

Medical Sciences of the National Research Council, had prevailed upon Dr. Paul to organize the meeting and to act as chairman.

Dr. Bayne-Jones commented that the problem of hepatitis had been one of the major preoccupations of the Army Epidemiological Board and, later, of the Armed Forces Epidemiological Board, ever since the disease gained new prominence with the epidemic of post-inoculation jaundice in 1942. He was pleased to see so many of the workers in this difficult field meeting together to re-evaluate the important and elusive problem of isolating and detecting the causative agent of hepatitis.

Dr. Paul then called on Drs. Bang and Werner Henle to present papers on their attempts to propagate hepatitis viruses in tissue culture. He asked Dr. Bang to introduce the subject with a general discussion of some variables in the destructive effect of viruses on cells.

II. Review of Attempts to Propagate the Agent of Human Hepatitis (both SH and IH) in Various Hosts

A. Tissue Culture

SOME VARIABLES IN THE DESTRUCTIVE EFFECT OF VIRUSES ON CELLS

DRS. FREDERIK B. BANG and G. O. GEY

Since the viruses of human hepatitis have not, as yet, been demonstrated to destroy cells in tissue culture, we chose to discuss the effect of several viruses on cells in tissue culture. From this we may be able to derive new ideas in the search for a destructive effect of the virus of hepatitis. We have divided this presentation into several sections, listing the variables which influence the destructive effect of viruses on cells. In giving examples concerning these effects we will concentrate on the results obtained in our own laboratories, since these results are most familiar to us. However, we recognize that many others are studying similar systems.

A. Type of culture

1. Studies of cells

a. **Species of animals as source.** The origin of the cells as to species of animal may not be essential. However, in general it is believed that viruses grow better in the cells of an animal which has been demonstrated to be susceptible. This is indicated by the susceptibility of Walker Rat Sarcoma 256 to the virus of Newcastle disease. This virus grows poorly in mammals, and thus we might expect that a rat tumor cell should not be susceptible. The cells are not destroyed by small amounts of the virus, but partial destruction may be brought about by inoculation of larger amounts (compare figs. 1 and 2). The recovery of the colony from this preliminary destructive effect is complete.

b. **Uniform cell strain.** Contrariwise, a cell line which has been derived from a susceptible host is not necessarily susceptible. The comparison of a normal rat cell strain (14PF) with a malignant derivative of that cell shows that the two cell lines vary greatly in susceptibility (1). The normal cell was almost completely resistant, whereas the malignant cell succumbed to minimal amounts of virus. Other cell lines derived from the same stock of rats showed intermediate susceptibility. The degree of malignancy of the cell has no constant relation to susceptibility to the virus, for embryonic cells are rapidly destroyed by the virus.

2. **Primary explants.** Some years ago the susceptibility of fibroblasts derived from the chick embryo to the virus of fowl pox was compared with the susceptibility of epithelial cells (2). Although fibroblasts supported the growth of fowl pox, no changes were found in these cells. On the other hand, the epithelial cells derived from the same embryo were destroyed by the virus, and thus it was not possible to maintain continuous cultures of epithelial cells with this virus.

3. **Organoids.** The use of tissue culture in the study of viruses has for the most part been limited to the study of separate cell lines or minced tissues observed in one way or another. An old discipline in tissue culture which involves the use of organoids to study the development of the specific developmental characteristics of organs (3) has not been adequately explored in tissue culture virus work. The submandibular gland of the mouse will develop much of its glandular structure in such cultures (4). Gaillard has shown that the human ovarian cortex and bits of human parathyroid can be cultivated for periods of two to four weeks in culture and that they will preserve many of the characteristics of the original organ (5). Since the very hallmark of virus activity is organ specificity, it seems obvious that organoids in tissue culture offer a real opportunity for virus study.

B. Source and type of material for inoculation

1. **Plasma, serum, white cells, or whole blood.** We have consistently used plasma or serum for the inoculation of cultures with the virus of hepatitis. This material is used because it has been demonstrated to be infectious for man by studies such as that of Dr. Murray on volunteers (6). However, examples are common in the field of viruses in which the presence of antibodies is not apparent when a mixture of virus and antibody is inoculated into one host, and yet the same mixture of virus and antibody may be neutral when inoculated into another host. Thus, we cannot be sure when a serum or plasma, known to be positive in man, is inoculated into cells in tissue culture that antibody has not prevented the infection of the cells. There are several ways in which one may improve the chances of infecting the cells. Table I illustrates some pertinent results obtained by Dr. Liu several years ago when he was working in our laboratory. It shows that Newcastle virus may be isolated from white cells even when antibody is present in the serum and

when the plasma or a mixture of whole blood may be negative for virus. We need to try to see whether inoculation of white cells from patients in the acute stage of hepatitis might not be a better source of infectious material.

TABLE I

LOG LD_{50} OF VIRUS RECOVERED FROM DIFFERENT BLOOD FRACTIONS OF CHICKENS INOCULATED WITH NEWCASTLE DISEASE VIRUS

	Days after inoculation						
	1	3	5	6	7	8	10
Plasma	1.2	1.8	0	0	0	0	0
Washed RBC	1.0	1.8	1.2	Un.*	0	0	0
Whole blood	1.6	2.0	1.2	Un.*	Un.*	0	0
Washed WBC	2.7	2.6	2.8	1.7	1.0	0	0
Neutralizing antibody vs. 100 LD_{50} virus	1/5	1/5	1/10	1/80	1/860	1/650	1/1600

*Un.—Undiluted specimen

2. **Liver biopsy.** Recent work by Weller (7) on the virus of chicken pox has indicated that this virus spreads within the infected culture by direct contact, but does not appear in the fluid. Thus, cells have to be exposed under particular conditions which bring infected and normal cells into close proximity. Liver biopsies from acute cases of hepatitis should offer a chance for direct infection of cells in tissue culture. Explants of chick embryo tissue which have been previously infected in the embryo with the virus of either influenza (8) or Newcastle disease (9) will give continued growth of the virus, and have made possible certain electron microscope studies of the development of the virus.

3. **Autopsy.** A similar technique might be applied to tissue from autopsies. These cells will grow readily if obtained a few hours after death of the host, and could be grown in juxtaposition with cultures of other cells such as embryonic liver.

4. **Strains of virus.** Dr. Henle has several times emphasized the need for a study of a variety of strains of hepatitis virus in the search for a destructive effect. We can illustrate this need by reference to the comparison of the growth of an avirulent strain of Newcastle disease virus with that of a virulent one in homologous chick cells and media (10). The virulent strain rapidly destroyed both fibroblasts and macrophages. In the complete absence of antibody, however, the avirulent strain slowly destroyed the fibroblasts and grew in cultures of macrophages for two months without producing any visible effect on the cell population.

C. Mode and time of inoculation

1. **Amount of virus inoculated.** The effect of the amount of virus inoculated may be illustrated by reference to the effect of our avirulent

strain of Newcastle disease virus on chick heart fibroblasts. Large amounts of the virus in undiluted allantoic fluid destroyed the explant within several days. However, it took some ten to 14 days to produce clear effects when minimal amounts of virus were inoculated (10^{-7} dilution).

2. **When should tissues or cells be exposed to virus?** We originally found in the electron microscope study of eastern equine encephalitis that cells heavily infected with the virus might be obtained when the explant was infected at the same time that it was set up in static cultures. However, if the cells were allowed to grow out first and were then infected, minimal amounts of virus were obtained even though the cells were destroyed. It is possible that this quantitative difference in the yield of virus has no direct bearing on the basic capacity of virus to destroy cells, but this relationship needs to be investigated if we are to understand the process and wish to obtain the best results.

3. **Exposure of cells.** Colonies may, of course, be established in fibrin clots or grown directly on the glass. It seems likely that a monocellular layer and a free exposure of cells to virus is preferable. However, many cells grow better in fibrin clots. The extent to which they may be protected from virus inoculation by growth within a fibrin clot is indicated by the appearance of a type of colony destruction which has been consistently obtained in our laboratory. Rings of destruction appear around the colony, while the central fragment is intact as if it had not been adequately exposed to virus.

4. **Predigestion with trypsin.** The digestion of bits of organs with trypsin for the establishment of uniform cell population is now a common procedure. It is likely that the present treatment with trypsin changes the cell's capacity to take in particles, and may influence the infection. In our original work on lymphopathia and the infection of human fibroblasts it was shown that cells previously exposed to trypsin are susceptible to the virus of lymphopathia (11).

5. **Centrifugation onto cells.** Recently, in connection with the study of hemorrhagic fever, it was thought necessary to be sure that the cells

TABLE II

SURVIVAL OF HELA CELL COLONIES ULTRACENTRIFUGED WITH EASTERN EQUINE ENCEPHALITIS VIRUS (CHICK EMBRYO SUSPENSION)

Fluid used	Virus added	Temp. ° C.	Force	Survival Pattern	
				Centrifuged	Uncentrifuged
20 per cent cord serum	10^{9}	36.5	81,000 g.	Gran. & degen. cells—48 hrs.	No degen. 48 hrs.
	10^{7}			Same as above	Sl. degen. 48 hrs.
	10^{4}			Severe necrosis—48 hrs.	50 per cent degen. 48 hrs.

were adequately exposed to a definite amount of virus. For this reason, a method of centrifuging virus onto cells in the ultracentrifuge was developed. The accompanying table (table II) illustrates, with the virus of encephalitis, that it is possible to place cells in such centrifuge tubes and to expose them to virus for an hour or more during highspeed centrifugation. The cells are destroyed if they have been inoculated with minimal amounts of virus, or recover if virus has not been present. These methods are being applied to the study of hepatitis.

D. Variations in conditions of growth

1. **Temperature.** In general, the studies of viruses in chick embryos have led to the belief that a temperature of 35° C. is optimal for virus growth. We may not, however, infer that such temperatures are the best for the growth of viruses in different kinds of tissue culture. We have recently found that the strain Rat Cell TSAT-72 will support the growth of eastern equine encephalitis virus at temperatures ranging all the way from 21° to 41° C. (13). Growth is more rapid at the higher temperature, but occurs at the lower temperature despite the poor appearance of the cells in the control culture. Furthermore, the avirulent strain of Newcastle disease virus which does not destroy cells at 35° C. produces destruction of all macrophages present in the culture when these are incubated at 41° C. These macrophages survive and grow well without virus at these higher temperatures (10) which are, of course, normal for the chicken.

2. **Nature of food.** The whole story of the growth requirements of viruses has just begun to be available to study since uniform cell strains have been infected with several viruses (1, 12, 13, 14). We have mentioned before our desire to grow the cells in media free of antibody, and for this reason many of our studies have utilized horse and rabbit serum. Dr. Enders' group has used human cells grown in bovine amniotic fluid. The usual tissue culture is grown in a clear serum which is optimal for growth of the cells, and the lipid content is usually minimal. We have no right to assume that this is the best method for the growth of a virus such as hepatitis. Liver cells are continually bathed by plasma which has recently picked up a large variety of fat and protein materials in the intestine.

3. **Rate of feeding.** Cells in tissue culture may be kept in a "good" state or have a poor appearance depending upon the rate at which the nutrients are replaced. If the fluid is replaced at infrequent intervals there is a rise and fall in a variety of plasma components which feed the cells. This is not similar to the situation present in the intact animals. Furthermore, most of the cultures consist of rapidly growing cells with a continuous replacement of medium which tends to increase the rate of growth. Such growth may perhaps be limited by the less frequent replacement of fluid and the use of a medium which more closely resembles that in the intact animals.

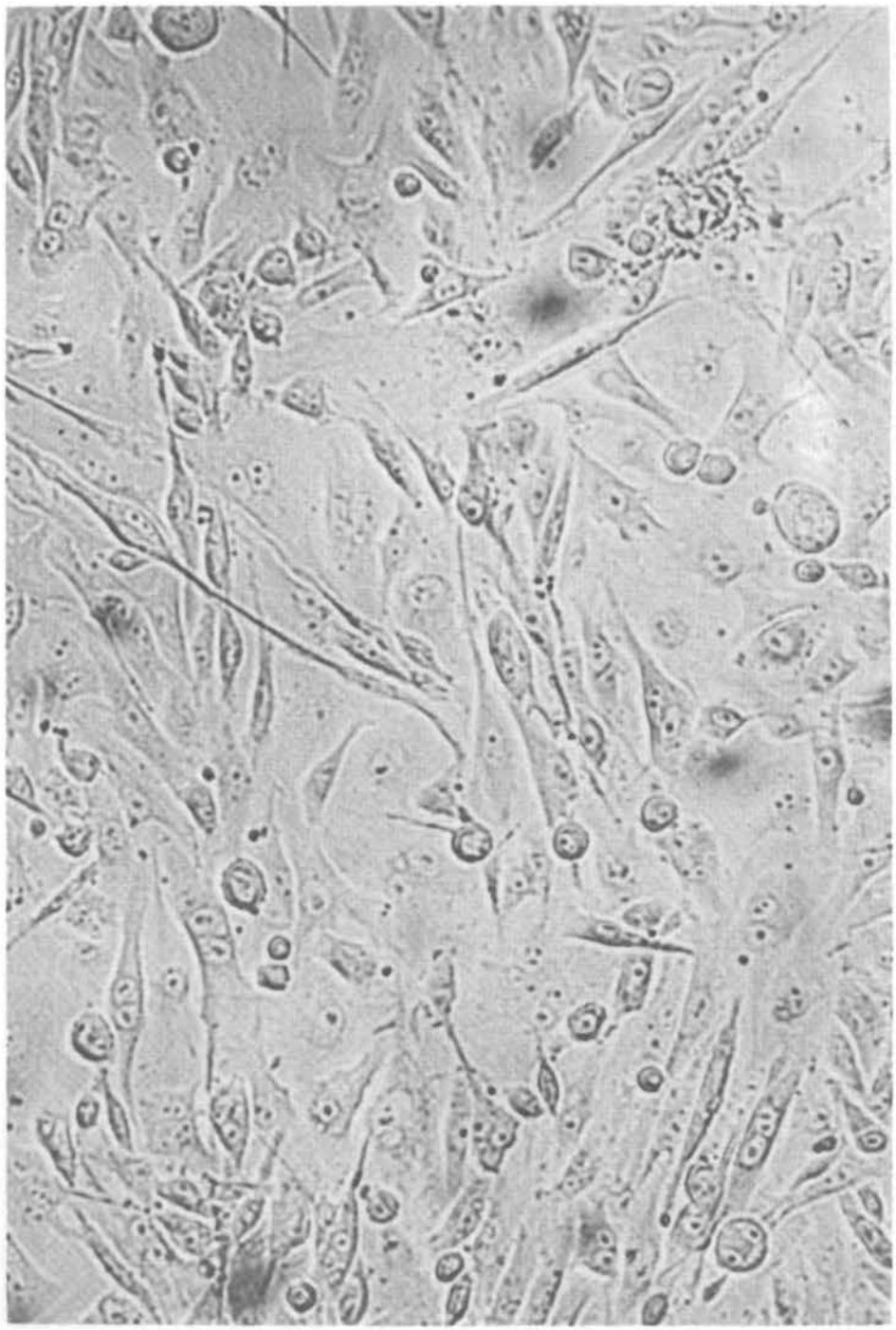


Figure 1. Walker Rat Sarcoma 256 in roller tube tissue culture, uninfected.

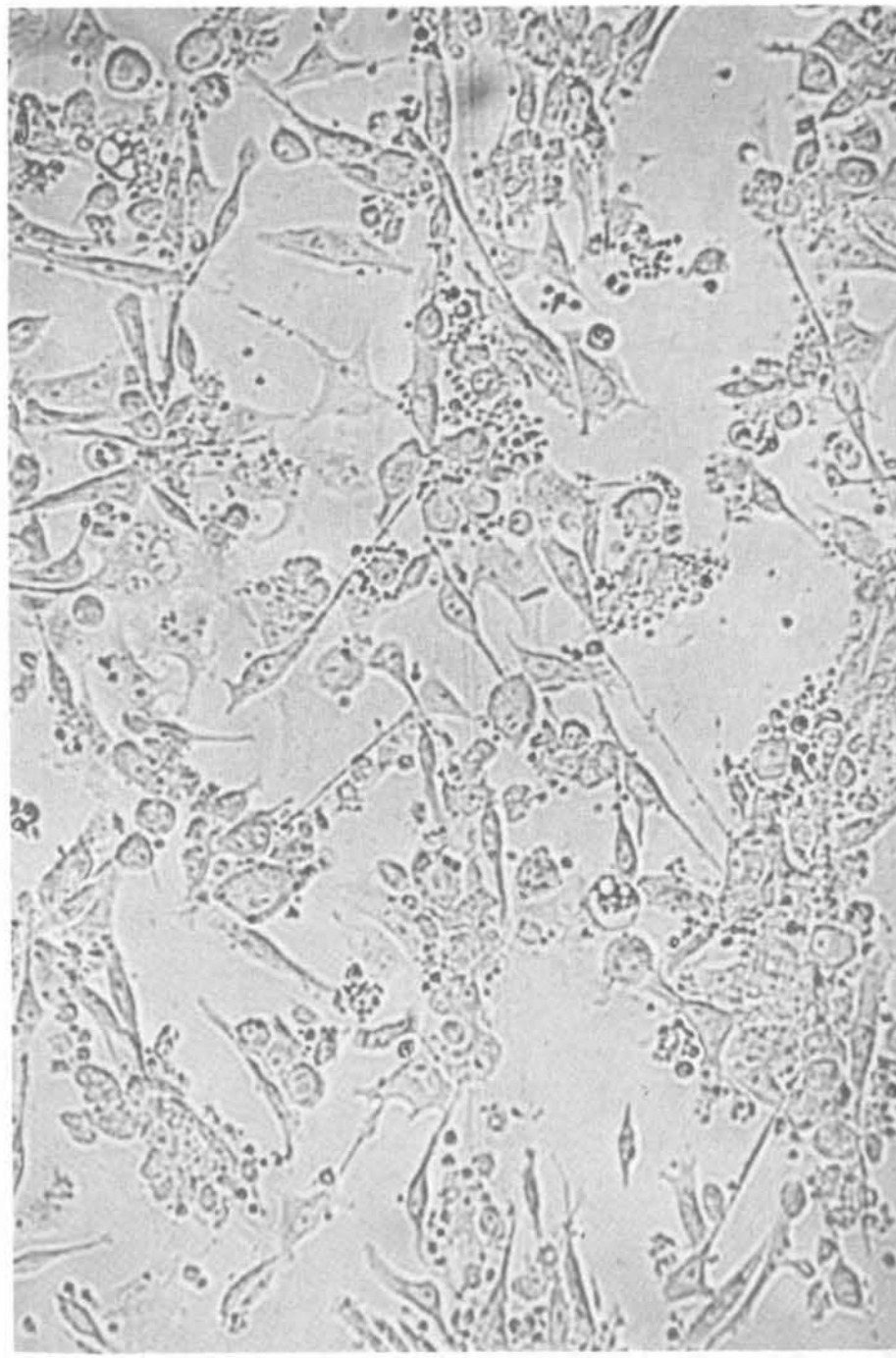


Figure 2. Walker Rat Sarcoma 256 in roller tube tissue culture exposed to undiluted allantoic fluid containing Newcastle virus.

4. **Partial anaerobiosis.** Cells may grow under relatively aerobic conditions such as are present in the use of roller tube, or they may grow in a drilled slide preparation in which very little oxygen is obtained. A difference in the susceptibility of cells to the different conditions—aerobic versus anaerobic—is indicated by some old experiments of ours on encephalitis virus. This needs greater exploration.

We hope that this summary will serve as a stimulus to individual work in the search for a destructive effect of the virus of hepatitis, for we believe that, despite the continued careful work on the part of several laboratories, only a beginning has been made.

References

1. Bang, F. B., and Gey, G. O.: Comparative susceptibility of cultured cell strains to the virus of eastern equine encephalomyelitis, Bull. Johns Hopkins Hosp. *91*:427-461, 1952.
2. Bang, F. B., Levy, E., and Gey, G. O.: Some observations on host-cell virus relationships in fowl pox: I. Growth in tissue culture; II. The inclusion produced by the virus on the chick chorio-allantoic membrane, J. Immunol. *66*:329, 1951.
3. Fell, H. B.: Histogenesis in tissue culture, cytology and cell physiology, ed. 2, edited by G. H. Bourne, London, Oxford U. Press, 1951, ch. X, p. 419.
4. Borghese, E.: The development *in vitro* of the submandibular and sublingual glands of *Mus musculus*, J. Anat. *84* (3) July, 1950.
5. Gaillard, P. J.: Growth and differentiation of explanted tissues. International Review of Cytology, edited by Bourne and Danielli, New York, Academic Press, 1953, p. 331.
6. Murray, R. G.: In present series.
7. Weller, T. H.: Serial propagation *in vitro* of agents producing inclusion bodies derived from varicella and herpes zoster, Proc. Soc. Exper. Biol. & Med. *83* (*2*); 340, 1953.
8. Murphy, J. S., Karzon, D. T., and Bang, F. B.: Studies of influenza A (PR8)-infected tissue cultures by electron microscopy, Proc. Soc. Exper. Biol. & Med. *78*:596, 1950.
9. Bang, F. B.: The development of Newcastle disease virus in epithelial and fibroblast cells in tissue culture, Bull. Johns Hopkins Hosp. *92*:291, 1953.
10. Bang, F. B., and Warwick, A.: A virulent and an avirulent strain of Newcastle disease virus in tissue culture, Fed. Proc. *13 (1)*:421, March 1954.
11. Gey, G. O., and Bang, F. B.: Experimental studies on the cultural behavior and the infectivity of lymphopathia venerea virus maintained in tissue culture, Bull. Johns Hopkins Hosp. *45(5)*:393, Nov. 1939.
12. Scherer, W. F.: The utilization of a pure strain of mammalian cells (Earle) for the cultivation of viruses *in vitro:* I. Multiplication of pseudorabies and herpes simplex viruses, Amer. J. Path. *29(1)*:113, Jan. 1953.
13. Scherer, W. F., Syverton, J. T., and Gey, G. O.: Studies on the propagation *in vitro* of poliomyelitis viruses: IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix, J. Exper. Med. *97(5)*:695, May 1953.
14. Stoler, M., and Gey, M. K.: Destruction of a human fibrosarcoma strain (A. Fi.) in tissue cultures following the growth of poliomyelitis viruses, Bull. Johns Hopkins Hosp. *92*:385, 1953.

ATTEMPTS TO PRODUCE DESTRUCTION OF CELLS BY HEPATITIS VIRUS IN TISSUE CULTURE

DRS. FREDERICK B. BANG AND ANNE WARICK

The search for a destructive effect of the virus of hepatitis has been undertaken, in general, in two ways. The first, and most direct, is to inoculate a variety of cells, particularly human, with material known to be positive by previous testing in man (1, 2). The second is to study the tissue culture requirements for the destruction of mouse cells by a virus known to produce rapid destruction of mouse liver cells. From such data we might plan better experiments dealing with human hepatitis.

The direct approach has been complicated by several things. In the absence of cellular destruction, it is not known whether or not the virus is actually present. Growth of other viruses may occur in cultures in which so little destruction is apparent that the effect of the virus cannot be brought out (3, 4). Destruction of cells, indistinguishable from that produced by viruses, may occur in the absence of known viruses. This destruction may be due either to contaminating viruses or to unknown variations in nutrition, or to both.

Table I includes all of the experiments which we have made using human embryo explants. Since these embryo explants were grown in human cord serum, it is likely that the tests were performed in the presence of some antibody. To escape this effect each embryo tissue

TABLE I

PRIMARY EXPLANTS OF HUMAN EMBRYO TISSUES IN ROLLER TUBES WITH INFECTIOUS HEPATITIS (AKIBA) AND SERUM HEPATITIS (MERRIAM) VIRUSES

Type of Tissue	Times Tested		Media	Time Followed
	IH	SH		
Liver	2	1 1	Human cord (4)* Chick serum	14-20 days
Muscle	1 1 1		Human cord (4) Chick serum Rabbit serum	15 days
Gut	2	1 1	Human cord (4) Chick serum	14-20 days
Kidney	1	1 1	Human cord (4) Chick serum	20 days

* The numeral 4 refers to the fact that these experiments were set up in four different cord sera (see text).

was placed in four separate cord sera. Each combination of tissues and cord serum was set up in duplicate. The pair of culture tubes was kept in this serum for the duration of the study. Two control roller tube cultures were maintained in the same serum. Although on one occasion destruction was seen in the infected tubes containing kidney and muscle and not in the controls, we have not as yet had the opportunity to transfer this destructive effect to new embryo cultures.

Because of our previous work with eastern equine encephalitis, in which different cell lines were found to have different susceptibilities to the virus (5), we have been interested from the beginning in the possible use of the variety of human cell lines available in Dr. Gey's laboratory. Table II shows the tests which have been done with two of the less frequently used cell lines. The A.Fi. strain has been shown to be susceptible to polio (6). The most extensive tests have been performed with the HeLa strain, derived from a carcinoma of the cervix (7). Table III summarizes the tests for susceptibility of this cell line. In none of these experiments have we obtained destruction of HeLa cells which could be reproduced on transfer to new cultures and which occurred in the absence of similar destruction of the control. Although this cell line will grow adequately at 34°C. in heterologous media, particularly horse serum, destruction at this lower temperature is nevertheless great enough in the controls constantly to cause difficulties in interpretation. However, the variety of factors possibly influencing the growth of cells in tissue culture, as outlined in the previous paper (8), means that there are many more experiments to be performed.

Because of the original reports of Henle (9) on the growth of both infectious hepatitis and serum hepatitis in chick embryos, we early

TABLE II

HUMAN TUMOR CELL LINES IN ROLLER TUBES WITH INFECTIOUS HEPATITIS (AKIBA) AND SERUM HEPATITIS (MERRIAM) VIRUSES

Type of Tissue	Times Tested		Media	Time Followed
	IH	SH		
A. Fi.	2		Human cord (4)*	4-34 days
	1	2	Chick serum	
	2	2	Difco 199	
Di Re		1	Chick serum	8-13 days
		1	Rabbit serum	
	1	1	Difco 199	

* The numeral 4 refers to the fact that these experiments were set up in four different cord sera (see text).

TABLE III

INOCULATIONS OF HUMAN CERVICAL CARCINOMA (HELA) WITH HEPATITIS VIRUS ROLLER TUBE EXPERIMENTS

Exp. No.	Media	Temp. (C)	Strain of Virus	No. of Tubes	Day After Explant When Inoculated	Days Held After Inf.
I	50% cord s.	37	IH*	6	23	21
II	25% chick s.	37	IH	4	2	10
III	Difco 199	37	IH	6	3	21
IV	Difco 199	37	IH Pass. fld. fr. III	5 5	3	19
V	25% cord s.	34	SH Merriam Krow	4 4	5	25
VI a.	50% horse s.	37	IH SH NIH-8	3 3	15	44
VI b.	50% horse s. 10% beef emb. ext. 40% B.S.S.	37	IH SH NIH-8	3 3	15	6
VII	50% horse s.	37	SH Fld. fr. VI	4	2	8
VIII	50% horse s.	34	SH Fld. fr. VI	6	2	72
IX a.	50% horse s.	34 37 41	SH NIH-8 Merriam	7 7	33	123 still held
IX b.	75% horse s.	34 37	SH NIH-8 Merriam	3 3	33	123 still held

* All IH virus used was from the Akiba pool furnished by Dr. G. S. Mirick. This was demonstrated in human volunteers to be positive for virus.

tested the susceptibility of explants from such embryos. These have been summarized in table IV. We have obtained no destructive effect which could be related to the virus inoculum. We have frequently seen destruction, and several times it seemed that this effect was transferred to new tubes. However, in our hands, primary explants of epithelium, which was the main cell type used, so frequently showed a variable amount of destruction that we were not able to interpret the moderate destruction found in the infected tubes.

The second part of our study has involved the study of Nelson's mouse hepatitis virus (10). Thin sections of the liver, as studied in the electron

TABLE IV

PRIMARY EXPLANTS OF CHICK EMBRYO TISSUES IN ROLLER TUBES WITH INFECTIOUS HEPATITIS (AKIBA) AND SERUM HEPATITIS (MERRIAM) VIRUSES

Type of Tissue	Times Tested		Media	Time Followed
	IH	SH		
Skin	8		25% chick serum	9-12 days
Liver	5	2	25% chick serum	5-14 days
Muscle	1		25% chick serum	12 days
Kidney	1		25% chick serum	12 days
Intestine	1		25% chick serum	9 days
Amnion	1		25% chick serum	12 days

microscope, show numerous particles about 35 μ in size. These have an internal structure, and have all the appearances of virus particles (figs. 1 and 2). Currently we are attempting to grow this virus in primary explants of mouse liver. Good cultures of these cells may be obtained by explant of newborn mouse liver into horse serum and beef embryo extract. These cells survived long enough so that the effect of the virus may be studied. Figure 3 shows such cells obtained from a primary explant.

References

1. Murray, R. G.: In present series.
2. Mirick, G. S.: Personal communication.
3. Bang, F. B., Levy, E., and Gey, G. O.: Some observations on host-cell virus relationships in fowl pox: I. Growth in tissue culture; II. The inclusion produced by the virus on the chick chorio-allantoic membrane, J. Immunol. *66*:329, 1951.
4. Bang, F. B., and Warwick, A.: A virulent and an avirulent strain of Newcastle disease virus in tissue culture, Fed. Proc. *13(1)*:421, March 1954.
5. Bang, F. B., and Gey, G. O.: Comparative susceptibility of cultured cell strains to the virus of eastern equine encephalomyelitis, Bull. Johns Hopkins Hosp. *91*: 427-461, 1952.
6. Stoler, M., and Gey, M. K.: Destruction of a human fibrosarcoma strain (A. Fi.) in tissue cultures following the growth of poliomyelitis viruses, Bull. Johns Hopkins Hosp. *92*:385, 1953.
7. Gey, G. O.: Personal communication.
8. Bang, F. B., and Gey, G. O.: In present series.
9. Henle, W., Harris, S., Henle, G., Harris, T. N., Drake, M. E., Mangold, F., and Stokes, J., Jr.: Studies on the agent of infectious hepatitis: I. Propagation of the agent in tissue culture and in the embryonated hen's egg, J. Exper. Med. *92*:271, 1950.
10. Nelson, J. B.: Acute hepatitis associated with mouse leukemia: I. Pathological features and transmission of the disease, J. Exper. Med. *96*:293, 1952.

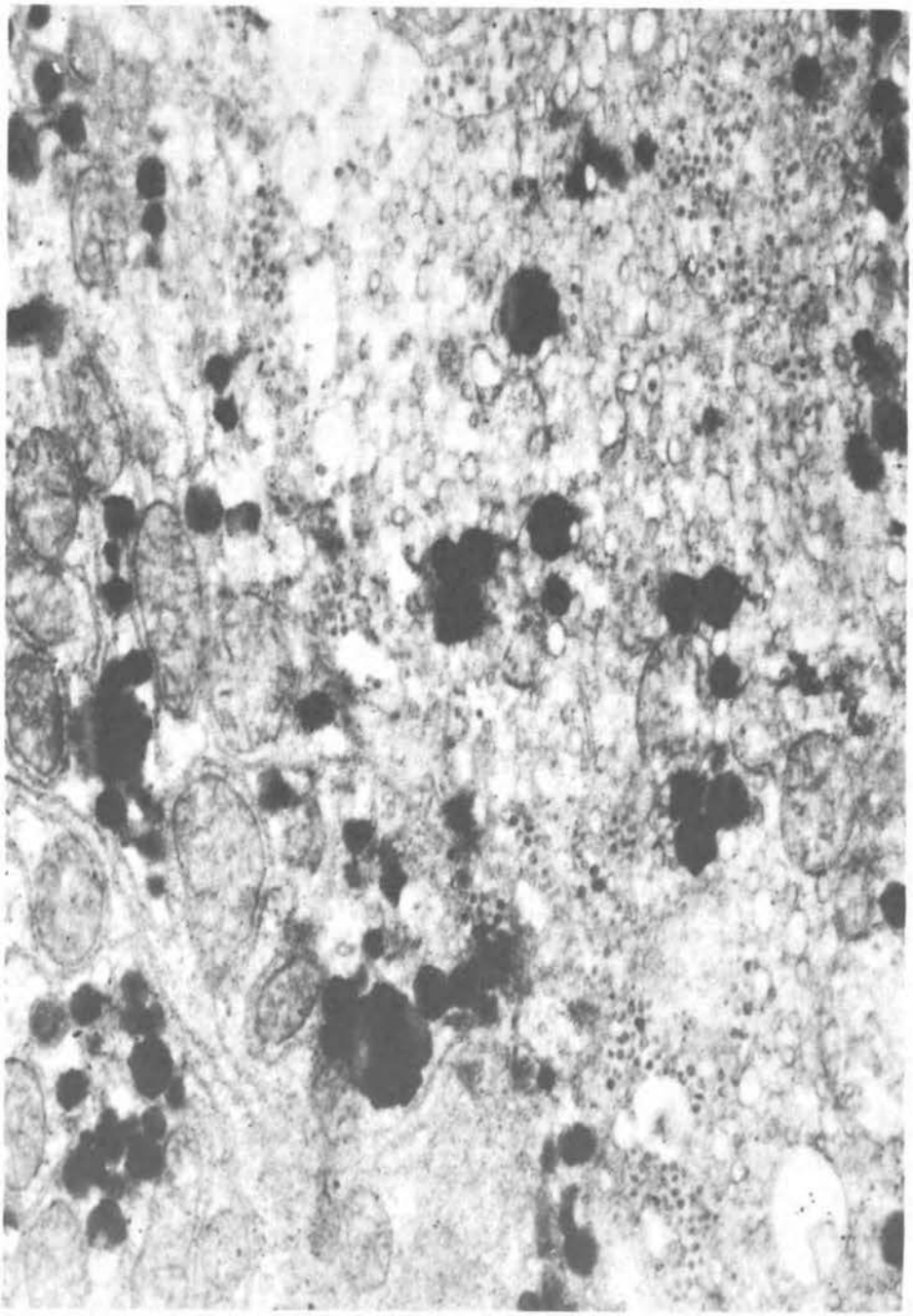


Figure 1. Section of liver taken from a 44-hour infection of mouse with Nelson's hepatitis virus. Small ovoid particles scattered throughout the tissue are presumed to be virus. Magnification 19,600 X.

Figure 2. Mouse liver infected with Nelson's hepatitis virus, showing capillary with virus extruded from liver cell into endothelial space. Magnification 19,600 X.

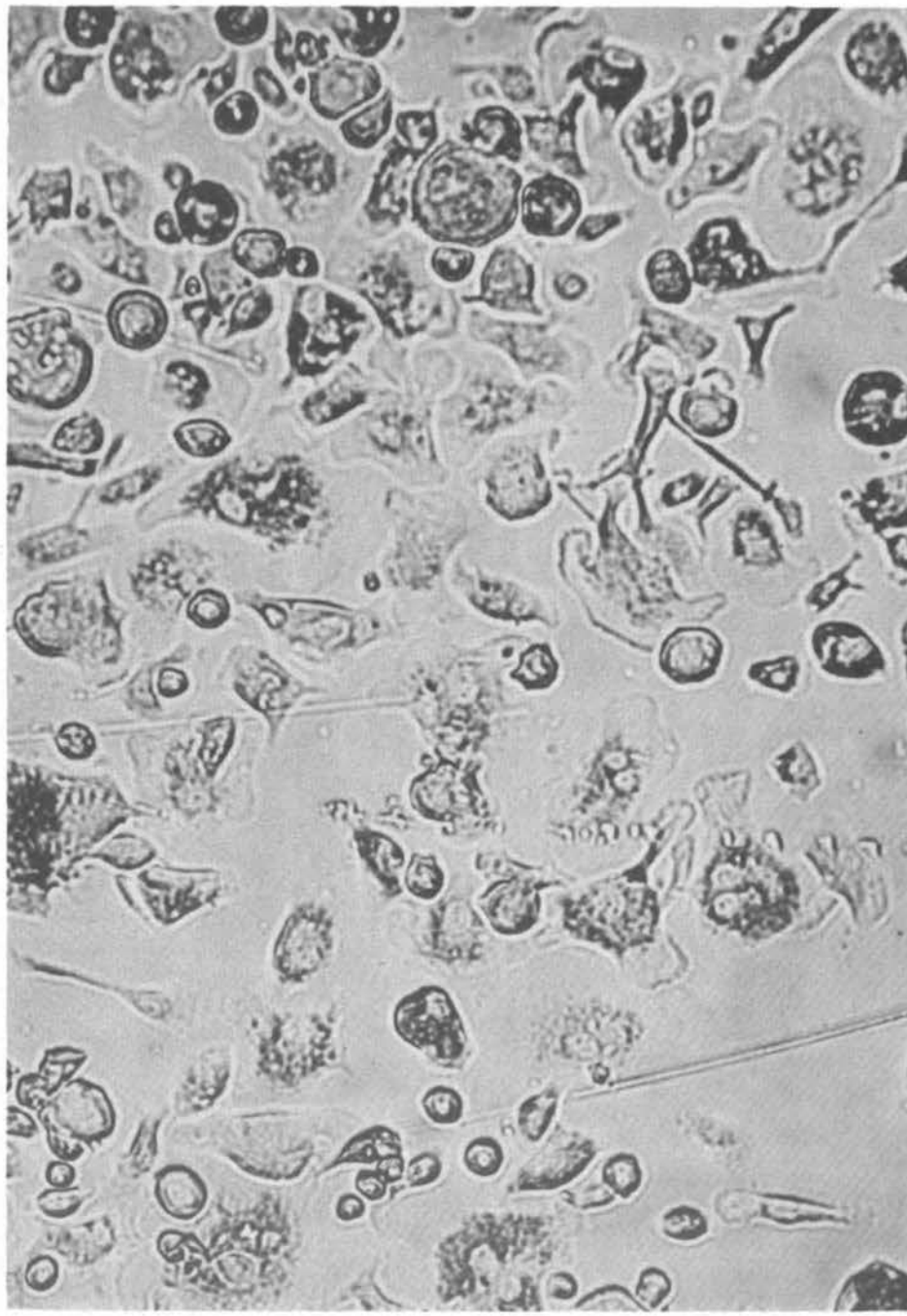


Figure 3. Primary explant of mouse embryo liver cultures in roller tube with horse serum.

SERUM HEPATITIS AND INFECTIOUS HEPATITIS: THE USE OF HUMAN CELL CULTURES, HELA STRAIN*

DR. JEROME T. SYVERTON

These studies were undertaken as an attempt to propagate the agents of infectious hepatitis and serum hepatitis in cultures of a stable human cancer cell, strain HeLa, Gey. The viral range of this human epithelial cell in continuous culture on glass has been demonstrated in this laboratory (1, 5-11) and other laboratories (3, 4) to encompass a wide variety of viruses infective for man and animals (1, 3-11). Thus, cells in continuous culture support propagation in vitro of the three types of poliomyelitis virus (8, 9); three immunologic types in the B group of Coxsackie viruses (1, 10, 11); the viruses of eastern equine encephalomyelitis, western equine encephalomyelitis, West Nile, St. Louis encephalitis, Japanese B encephalitis, pseudolymphocytic choriomeningitis, herpes simplex, pseudorabies, and vaccinia (5-7, 10, 11). Thirteen of the 15 viruses regularly destroy strain HeLa cells; infection by St. Louis encephalitis virus and Japanese B encephalitis virus results in propagation of the virus, but uncommonly causes cellular destruction (6). Two recently discovered agents, the A.D. agent (4) and the RI-67 virus (3), as yet incompletely characterized, are cytopathogenic for HeLa cells in culture.

MATERIALS AND METHODS

HeLa Cell Strain, Gey (2).—The methods applied routinely in this laboratory for cellular cultivation, for the preparation of cells for transfer, for cellular enumeration, and for the preparation of cell cultures have been described fully (9). The medium for cellular cultivation consisted of human adult serum, 40 parts, chicken embryonic extract, 2 parts, and Hanks solution, 58 parts. The cells employed were cultured in tubes containing from 60,000 to 100,000 cells. The cellular cultures were washed thrice to remove all human serum and maintained thereafter by maintenance solution, 80 parts, and chicken serum, 20 parts, with incubation at 36° C. The supernatant fluid was replaced once or twice weekly, as determined by changes in pH.

Materials used for inoculation.—Five samples of serum presumed to contain the agent of serum hepatitis were made available by Dr. Roderick Murray. These samples were identified as NIH-8, serum with a take rate of 52 per cent for all types of hepatitis; "Merriam" plasma, from a volunteer within the first few days of illness resulting from infection with NIH-8 material; L-181 (7-25-52), taken from a donor who developed hepatitis later; L-204 (12-5-52), and L-207 (12-5-52) drawn

* This study was supported in part by a grant from the National Institutes of Health, Public Health Service.

within the first few days of illness from two recipients who had received infectious plasma from a carrier.

The test samples of serum, feces, liver, and spleen presumed to contain the agent of infectious hepatitis were of unknown infectiousness. Drs. Cecil Watson and F. W. Hoffbauer made available the specimens identified as M-2000, M-2001, M-2002, M-2003, and 51F2; the other specimens came from patients hospitalized with presumptive infectious hepatitis and from a fatal case of hepatitis (A Lo). These materials had been kept for variable periods at —50° to —60° C. Additional data for the test materials are contained in tables I and II.

Preparation of materials for inoculation.—The aliquots from serum samples were used directly. The fecal samples and tissues were prepared by a procedure applicable for the recovery of virus from any material containing a mixed microbial flora, as described previously for the isolation of poliomyelitis virus from fecal specimens (9).

Results

The procedure described elsewhere (9) for the culture of strain HeLa human cancer cell on stationary glass surfaces were applied to the isolation of the agents of serum hepatitis and of infectious hepatitis. The specimens employed were limited to serum samples from cases of serum hepatitis, and to samples of serum, feces, liver, and spleen from cases of infectious hepatitis. The data and results for a wide variety of pilot experiments are given in tables I and II.

TABLE I

RESULTS OF EXPERIMENTS H12-16

Attempts to Establish Serum Hepatitis Virus (SHV) in HeLa Cell Cultures

Exp. No.	Test Material		Data Relating to Cellular Cultures			Interpretation	
	Patient	Serum	No.of Passages	Passage Interval	Days Observed	Cyto-pathology	Propagation
H-12	NIH-8	0.1 ml.	3	14-20	35	0	Not Proved
R1-H12	NIH-8	0.1 ml.	3	10-30	64	±	Not Proved
R2-H12	NIH-8	0.1 ml.	1	67+	67+	0	Not Proved
H-13	L-181	0.1 ml.	4	2-25	31	0	Not Proved
R1-H13	L-181	0.1 ml.	2	15-50	50	0	Not Proved
R2-H13	L-181	0.1 ml.	1	67+	67+	0	Not Proved
H-14	L-204	0.1 ml.	1	0	31	0	Not Proved
H-15	L-207	0.1 ml.	1	0	31	0	Not Proved
H-16	Merriam	0.1 ml.	2	14-33	52	0	Not Proved

TABLE II

RESULTS OF EXPERIMENTS H1-H11, H18-H20, H-23
Attempts to Establish Infectious Hepatitis Virus (IHV) in HeLa Cell Cultures

	Test Material		Cellular Cultures			Interpretation	
Exp. No.	Patient	Inoculum, 0.1 ml.	No. of Passages	Passage Material	Days Observed	Cyto-pathology	Propagation
H-1	Ke Mo	Feces	4	6-23	44	±	Not Proved
H-2	Ke Mo	Feces	4	4-24	45	0	Not Proved
H-3	M-2001	Serum	1	0	30	0	Not Proved
H-3	M-2001	Feces	2	10-19	33	±	Not Proved
H-4	M-2000	Serum	1	0	30	0	Not Proved
H-4	M-2000	Feces	4	9-25	80	±	Not Proved
H-5	M-2002	Serum	1	0	15	0	Not Proved
H-5	M-2002	Feces	2	10-22	34	0	Not Proved
H-6	M-2003	Feces	3	2-30	39	±	Not Proved
H-7	Sc-150	Feces	4	7-21	56	±	Not Proved
R1-H7	Sc-150	Feces	3	7-17	49	±	Not Proved
R1-H7	Sc-150	Serum	3	14-17	54	0	Not Proved
H-8	D-27	Feces	1	0	18	0	Not Proved
H-9	Sc-155	Serum	1	0	28	0	Not Proved
H-10	Sc-164	Serum	3	10-27	77	±	Not Proved
H-11	51F2	Feces	3	4-34	57	±	Not Proved
H-18	Richert	Serum 8-d	2	3-7	10	0	Not Proved
H-19	A Lo	Spleen	1	61+	61+	0	Not Proved
H-20	A Lo	Liver	2	4-61	61+	0	Not Proved
H-23	M-2040	Feces	4	4-61	61+	0	Not Proved

The data contained in table I show that five samples of serum presumed, on good evidence, to contain the agent of serum hepatitis failed to cause evident cytopathology in HeLa cell cultures.

Serum and fecal samples from 13 patients with clinical infectious hepatitis did not prove cytopathogenic in HeLa cell cultures (table II). Similarly, the liver and spleen from the fatal case was without cytopathologic effect.

The salient fact is that none of the experiments gave evidence of success.

DISCUSSION AND SUMMARY

Cultural studies in vitro were carried out by the employment of a stable human cancer cell, strain HeLa (Gey), in continuous culture on glass in attempts to demonstrate evidence for propagation in vitro of the agents of infectious hepatitis and of serum hepatitis. The specimens util-

ized for study consisted of serum, feces, liver, and spleen. Twenty unsuccessful experimental attempts were made to demonstrate cytologic changes in HeLa cell cultures by employing materials presumed to contain infectious hepatitis virus. Similarly, no evidence for a cytopathogenic effect was obtained in nine attempts to establish serum hepatitis virus in HeLa cell cultures. Since propagation of viruses may occur without cytopathology, these studies are inconclusive; the results of infectivity tests of the tissue culture fluid by transfer to man or to other susceptible living hosts are essential to a reasonable interpretation.

The presumed agents of hepatitis were kept without transfer for more than 60 days in cellular cultures under continuous cultivation. Factors altered in an attempt to bring about infection of HeLa cells were (a) the time the inoculum was permitted to stay continuously in contact with cells, (b) incubation of the test material with the original cellular population for a prolonged period of time, (c) variations in the interval in days between successive transfers, and (d) the total number of transfers.

Fecal suspensions may contain material other than viruses which is "toxic" for cells in culture. The cytologic effects produced by four of the fecal samples fell into this category; the effects of other samples may have resulted from slight toxicity, but the cellular changes suggested otherwise. However, since evidence of cytopathologic effect did not persist for more than three passages, the findings of all experiments were assumed to be negative. It is recognized that the results of these pilot experiments should not be interpreted as negative without ultimate use of susceptible hosts for tests of the infectivity of the issue culture fluids.

References

1. Crowell, R. L., and Syverton, J. T.: The cytopathogenic effect of Coxsackie viruses in human epithelial cell cultures, Fed. Proc. *13*:49, 1954.
2. Gey, G. O., Coffman, W. D., and Kubicek, M. T.: Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium, Cancer Res. *12*:264, 1952.
3. Hilleman, M. R., and Werner, J. H.: Recovery of new agent from patients with acute respiratory illness, Proc. Soc. Exper. Biol. & Med. *85*:183-188, 1954.
4. Rowe, W. P., Huebner, R. J., Gilmore, L. K., Parrott, R. H., and Ward, T. G.: Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture, Proc. Soc. Exper. Biol. & Med. *84*:570-573, 1953.
5. Scherer, W. F., and Syverton, J. T.: The viral range *in vitro* of a malignant human epithelial cell (Strain HeLa, Gey): I. Multiplication of herpes simplex, pseudorabies, and vaccinia viruses. In press.
6. Scherer, W. F., and Syverton, J. T.: The viral range *in vitro* of a malignant human epithelial (Strain HeLa, Gey): II. Studies with encephalitis viruses of the eastern, western, West Nile, St. Louis, and Japanese B types. In press.
7. Scherer, W. F., and Syverton, J. T.: The viral range *in vitro* of a malignant human epithelial cell (Strain HeLa, Gey): III. Studies with pseudolymphocytic choriomeningitis virus. General discussion. In press.

8. Scherer, W. F., Syverton, J. T., and Gey, G. O.: Studies on the propagation *in vitro* of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (Strain HeLa) derived from an epidermoid carcinoma of the cervix, J. Exper. Med. *97*:695-710, 1953.

9. Syverton, J. T., Scherer, W. F., and Elwood, P. M.: Studies on the propagation *in vitro* of poliomyelitis viruses. V. The application of strain HeLa human epithelial cells for isolation and typing, J. Lab. and Clin Med. *43*:286-302, 1954.

10. Syverton, J. T., and Scherer, W. F.: The multiplication of viruses other than poliomyelitis in a stable strain of human epithelial cell (Strain HeLa), Bact. Proc., pp. 42-43, 1953.

11. Syverton, J. T., and Scherer, W. F.: The application of mammalian cells in continuous culture for assays in virology, Ann. N. Y. Acad. Sci. In press.

TISSUE CULTURE STUDIES RELATED TO HEPATITIS

DR. WERNER HENLE

It has been and will continue to be the aim of these studies (a) to collect or develop, and to maintain in tissue culture lines a variety of cells of human origin, to work out routine procedures for explanting certain human tissues; and (b) to expose these cultures to a large number of strains of hepatitis virus after suitable conditions for growth and maintenance of the cells have been established. This approach can be considered, therefore, as consisting of (1) straight tissue culture studies, and (2) virus studies. The pertinent problems and results obtained to date will be discussed under these headings.

1. **Straight tissue cultures.** Initially, the explant method was largely employed, with embedding of the tissue fragments in plasma clots. Although it was found relatively simple to grow epithelioid cells from the livers, hearts, and lungs of infant mice, difficulties were encountered in obtaining similar results with human fetal tissues as well as with specimens secured at surgery or early autopsy. Furthermore, the irregular supply of human tissues made it difficult to gain experience with culture techniques and to investigate systematically the conditions satisfactory for growth of the cells as well as for virus studies. When HeLa cells became available it was thought that these offered greater promise for initial experimentation.

(a) Studies on HeLa cells. No great difficulties were encountered in growing and maintaining these cells in the laboratory under the prescribed conditions. However, in view of the ultimate aim of establishing infection with hepatitis viruses, it was thought desirable to grow the cells in bottles in the absence of human serum, in order to avoid the presence of antibodies to hepatitis virus which might have been retained in the cultures despite washing of the cells. Furthermore, it was deemed necessary to maintain the cells in good condition for long periods after distribution to test tubes. Both aims appear to have been achieved. HeLa cells had been adapted to grow in horse serum and had been carried through 20 passages in bottles without loss in yield. Culture tubes prepared with horse serum growth medium remained clear and in

excellent condition for periods up to three weeks. However, the strain of cells which had been obtained from the Microbiological Associates underwent certain changes on further passages: periods of increasingly rapid multiplication were followed by partial or nearly complete destruction; the cultures were built up again from a few surviving cells, only to go into the same cycle once more. The cells were less susceptible to poliomyelitis viruses, but possibly showed greater susceptibility to other agents. A new line obtained from Dr. Scherer is now in use.

(b) **Studies on other cell lines.** The A.Fi. strain of fibroblasts, derived from a fibrosarcoma, is available and similar attempts to grow the cells in the absence of human sera will be started. It is also anticipated that other lines of cells will be obtained from other laboratories in the near future. At the same time it is planned to return to human tissues and to establish routine procedures for explants and for possible passage of the outgrowing cells. The necessary steps have been taken to receive specimens from various gynecologic, obstetric, and surgical services.

2. **Virus Studies.** These have intentionally been held in abeyance in order not to waste valuable materials before the tissue culture techniques were considered sufficiently established. However, certain aspects can be reported here.

(a) **Hepatitis virus collection.** Since the volunteer work here and elsewhere has largely been discontinued, ready sources of *known* infectious materials in large quantities are no longer at hand. Those collected in the past have been nearly exhausted in previous volunteer studies, so that little material is left. On the other hand, there are still available a number of untested, *potentially* infectious specimens which will form part of the collection. These represent only a few different strains of virus. It is conceivable, however, that ultimate success in adapting hepatitis viruses to tissue cultures may depend on using high-titer materials containing a particular strain or mutant. For these reasons it is considered essential to collect potentially infectious materials from various geographic areas, and particularly from severe epidemics or sporadic cases. Although various agencies have expressed their willingness to cooperate in such a collection, the difficulties in organizing and financing the enterprise were found insurmountable, and in consequence our laboratory must assemble its own collection. Accordingly, the following two outbreaks of infectious hepatitis were investigated with the primary purpose of collecting appropriate specimens.

Mercersburg, Pennsylvania. A circumscribed outbreak of infectious hepatitis (IH) occurred in a rural school, in which the disease was largely spread by contact and initially involved only students of one grade. Their siblings subsequently introduced the disease in other grades. The close observation of contacts permitted the detection of early signs of infection and thus several specimens (blood and stool) were obtained in the pre-icteric stage.

Mexico. In late January, 1954, word was received from Dr. Carlos Campillo in Mexico City that a severe epidemic of IH with an unusually high mortality was in progress. Consequently, Dr. Mary N. Crawford, on the staff of the Virus Diagnostic Laboratory, was sent to Mexico. It turned out that in addition to the IH epidemic, serum hepatitis (SH) was involved in one laboratory outbreak. Since reporting of the disease became compulsory only in February, 1954, no accurate data on the incidence of IH have become available. However, of 80 cases admitted to the Children's Hospital, 12 died. The high mortality was apparently not due to economic or nutritional factors since all economic strata were involved, including some children of physicians. Some children died suddenly with encephalitic symptoms, yet autopsy revealed fulminant hepatitis. Although it has been difficult to obtain rapid information concerning fresh cases or fatal cases, Dr. Crawford ultimately succeeded in collecting several satisfactory specimens.

Outbreak of hepatitis in the Zapata Laboratory. This laboratory is responsible for the production of gamma globulin in Mexico, using the ethanol precipitation method. It has a staff of 86 professional, administrative, and technical workers. A case of hepatitis was found on September 20, 1953, and a second early in October. These were presumed to be part of the IH epidemic and, therefore, gamma globulin was given to some of the technical staff on October 6-8, and to another group on October 30. Of a total of 68 workers injected with gamma globulin, 35 developed hepatitis after long incubation periods, with four deaths. No cases were noted among those not receiving gamma globulin, except for the first two cases. There were no cases among family contacts. Of the two batches of gamma globulin involved, one had apparently been used on a large scale in the prophylaxis of poliomyelitis without ill effects. Several of the professional and administrative staffs either injected themselves with their own needles and syringes, or were injected by their own physicians, and showed no evidence of hepatitis. The available information indicates, then, that SH was involved in this outbreak, and that it was clearly related to the injections. However, it has been shown that gamma globulin *per se* could not be considered responsible, so it was most likely that improper techniques were used in administration of the gamma globulin. Several serum specimens collected from these patients by Dr. Campillo and by Dr. Crawford are at hand.

(b) **Studies with agents other than hepatitis viruses.** It is hoped that one or another strain of the hepatitis viruses will ultimately produce cytopathologic effects in one of the tissue culture lines. However, it is conceivable, too, that the viruses may grow without causing such effects. In that case, it might be possible to detect their presence by interference with other viruses. For this reason, attempts were made to adapt to the established tissue culture lines a number of unrelated viruses with widely divergent potentialities and tropisms. It was found in earlier experiments that influenza and Newcastle disease viruses exerted a cytopathologic effect upon epithelioid cells grown from infant mouse livers. More

extensive studies were carried out with HeLa cells. In addition to the poliomyelitis viruses, vaccinia and herpes simplex could readily be passed in series in HeLa cells, and comparative titrations in these cultures and on the chorio-allantoic membrane gave comparable results. Several strains of Coxsackie viruses of groups A and B likewise produced cytopathologic effects. One strain of Coxsackie B virus from stool suspensions was directly isolated in HeLa cells, and it produced a cytopathologic effect on first passage. This strain induced lesions in infant mice on second passage only. Three different strains of Newcastle disease virus have readily produced cytopathologic effects; comparative titrations in HeLa cells and in eggs gave only slightly higher titers in the latter. When infected allantoic fluid diluted to 10^{-8} (< 100 EID_{50}) was inoculated, the tissue yielded 10^{6} EID_{50} in 48 hours or less, indicating multiplication of the agent. Growth curves indicated that virus was liberated from the cells well in advance of cell destruction. Although influenza virus is said not to produce cytopathologic effects in HeLa cells, it was found that all four strains tested (PR8, F99, L_{247}, and Lee) did so in low dilutions (up to 10^{-3} dilution of allantoic fluid). However, the virus could not be passed in series and, furthermore, virus inactivated by ultraviolet light to the extent that it still caused interference in eggs also produced cytopathologic effects, although higher concentrations (10^{-1}) of this inactivated agent were required. Thus, the results indicate that a "toxic effect" had occurred. The active virus caused development of detectable hemagglutinins. An "incomplete cycle" of multiplication may therefore occur in HeLa cells, comparable to that which Schlesinger observed in the central nervous system of mice upon intracerebral injection of influenza virus.

Thus, a number of viruses of diverse activities are available for study of interference by hepatitis virus in HeLa cells. It is proposed to increase the number of agents by at least two: an encephalitic virus and the 17D strain of yellow fever.

(c) **Studies with hepatitis viruses.** No concerted effort to adapt hepatitis viruses to tissue cultures have been made as yet, for the reasons outlined above. A few attempts at passage were made in mouse liver epithelioid cells and human liver cultures (fibroblasts) without apparent effects. Three passage series with eight samples of IH virus were carried out in HeLa cells grown on human sera but maintained on horse serum, with five transfers made from each at intervals of three or four, seven, and 14 days. Lesions were not obtained in these cultures. These studies will now be repeated and extended to include additional hepatitis virus strains in cells grown on horse serum. In addition, the infected cultures will be tested for the development of interference with the viruses described above. One line of HeLa cells has been grown in the presence of SH serum. Tubes were prepared from bottles of the second passage of this material, and these were used for an interference experiment against ten to 100 tissue culture doses of the viruses listed in section (b). No interference was apparent.

DISCUSSION

Dr. Paul then invited discussion of the papers concerned with tissue culture.

Dr. Smadel asked Dr. Syverton to clarify a technical point in his presentation. If phenol red was added to the cultures as an indicator, how often were changes in pH observed during the life of the cultures?

Dr. Syverton answered that the pH indicator proved useful in three ways: (1) Approximately one of 12 samples of test fecal material, on transfer to HeLa cell cultures, resulted in an abrupt change in the indicator, usually reflecting acidity. Sodium bicarbonate was added to these cultures to counteract the acidity, but the effect of the change in pH on either culture or contained virus was not known. (2) Active metabolism of the HeLa cells in culture results in progressive acidity over a period of from three to five days. When this occurs, the maintenance medium must be replaced, or sodium bicarbonate added to restore the pH to 7.4. (3) Contamination from whatever source results commonly in a very abrupt change in pH. Evidence for the presence of contamination is readily determined by direct bacteriological examination and culture.

Dr. Enders requested that Dr. Bang describe more fully his technique of growing liver cells, since previous attempts at culture of these cells had so often been unsuccessful.

Dr. Bang stated that he had not meant to give the impression that he had liver cells growing continuously. The liver cells which he had shown in his illustration were from a primary explant taken from a mouse embryo, and tumor cells had been added somewhere along the line. By growing such primary explants in horse or rabbit serum, liver cells had been propagated in about 50 per cent of the experiments. The particular batch of liver cells illustrated in figure 3 of his paper had grown out well, and the growing culture still seemed to consist of cells derived from the primary liver explant rather than an overgrowth of macrophages.

Dr. Enders asked whether Dr. Bang had found any way of suppressing or limiting the growth of fibroblasts. He had found in his cultures, as had many others, that fibroblasts overgrew most of the other cells.

Dr. Bang replied that he had had the same difficulty with fibroblasts in only about half of his mouse liver cultures, but in the remainder, fibroblasts had not been a major problem. This might have been related to the fact that the tissues were obtained from mice which were only three to four days old.

Dr. Enders stated that he had been using human embryonic cells rather than mouse cells. He wondered whether the addition of cortisone might be helpful in promoting the growth of liver cells alone, since cortisone supposedly has the capacity to suppress the growth of fibroblasts more than it suppresses epithelial cells.

Dr. Bang replied that he had not used cortisone.

Dr. Gertrude Henle stated that she had added cortisone to several HeLa cell cultures without noticeable effect.

Dr. Enders then noted that Dr. Werner Henle had mentioned the development of resistance to viruses by HeLa cells after they had been cultured for a time under different conditions. Dr. Enders also had noticed the late development of resistance in a line of mixed cells from human embryo skin; after five or six months, these cells had become almost completely resistant to poliomyelitis, even when large quantities of virus were added. This development of resistance in the culture probably reflected a selection of resistant cells which had been present in the original explant.

Dr. Werner Henle added that the development of resistance to viruses by HeLa cells had come to his attention only recently. In the line he had studied, the poliomyelitis virus had not destroyed all of the cells. There might well have been destruction of some of the cells with continued good growth of the survivors.

Another phenomenon he had observed was that before the cells became resistant, there was always a fast upgrowth, which was then followed by a return to the previous slower rate of growth. He asked Dr. Syverton whether he had had experience with such cells.

Dr. Syverton answered that he had observed cells from the virus-resistant HeLa cell strain which Dr. Shields had received for study from a commercial laboratory. From two to four days after addition of poliomyelitis virus, these cells showed a general delay in growth. After this time, about 25 to 50 per cent of the cells remained viable and grew out to form new cellular islands, which then grew out completely and coalesced. He did not know whether this represented survival of an originally resistant strain of HeLa cells, presumably a mutant, whether the cells had been inadvertently mixed with another line of cells, or whether it was the result of some change that occurred in the cells during culture. Cell lines originating in Dr. Syverton's laboratory and now distributed to many other laboratories had not shown this development of resistance. Since the resistant line from the commercial laboratory had originally been developed from cells obtained for culture from at least four laboratories, the precise reason for the emergence of the resistant strain was not known. It was possible that the strain might be maintained for experimental purposes, since it responded fully to a new group of respiratory viruses. However, since this commercial firm had obtained new cells from Dr. Syverton's laboratory, the problem of a resistant HeLa strain no longer existed.

Dr. Paul asked Dr. Alice Moore to describe the attempts made in her laboratory at Memorial Center to cultivate hepatitis virus in tissue culture.

Dr. Moore said that the experience with hepatitis virus in her laboratory had been rather limited. Dr. Noyes had been able to grow human liver cells obtained from surgical specimens, and had then added to them

material containing human hepatitis virus obtained from Dr. Murray. No differences were noted between the control and inoculated cultures during ten-day to two-week periods of observation. All of the cultures deteriorated after this time.

In addition, human hepatitis material was added to cultures of five human tumors, and no effects were noted in the growth or histology of the tumors. Mouse ascitic tumors were also mixed at different temperatures with hepatitis material and then inoculated subcutaneously, but no significant inhibition of tumor growth occurred.

Dr. Bang asked whether Dr. Moore's tissue cultures were grown in human media or in non-human media free of hepatitis antibody.

Dr. Moore replied that the human serum had been removed from the cells and they were then grown in a maintenance medium.

Dr. Bayne-Jones asked whether Dr. Moore had attempted to transplant human liver cells to animals, perhaps in the anterior chamber of the eye, according to the technique of Dr. Harry Greene.

Dr. Moore said that the work at Memorial Center with heterologous transplants had not included such attempts with liver tissue.

Dr. MacCallum asked if Dr. Moore had tried to "butter" human liver cells onto the membranes of the embryonated hen's egg.

Dr. Moore replied that she had not.

Dr. Sabin wondered whether anyone had added diluted hepatitis serum to tissue cultures, as well as undiluted serum. This seemed of potential value in view of past experience with poliomyelitis viruses. Some strains of poliomyelitis virus would exert no cytopathologic effect in the original 10 per cent suspension, but a remarkable zone phenomenon appeared with dilution. The 10^{-1} concentration was negative but the 10^{-2} and 10^{-3} mixtures, and often even the more dilute suspensions, were positive. Dilution might have a double effect: it could eliminate an interfering virus population, and it could eliminate antibodies. Since so many negative results had already been obtained with undiluted sera, it seemed unlikely that the diluted sera would be effective, but the attempt might be worthwhile.

He also referred to Dr. Werner Henle's proposal that the interference phenomenon be used as a method to detect invisible propagation of the hepatitis virus. In Dr. Sabin's laboratory, it had been noted that Japanese B encephalitis multiplied to a very high level (10,000,000 mouse infectious doses) without producing cytopathologic effects on a culture of explanted monkey kidney cells.

Dr. Werner Henle asked whether Dr. Syverton had taken the HeLa cells in which Japanese B encephalitis virus had multiplied without cytopathologic effects, and then exposed them to other viruses to see if the resistance carried over.

Dr. Syverton stated that he had not exposed such cells to hepatitis virus. Conversely, however, he had routinely taken cells which had survived as long as 60 days of exposure to hepatitis virus and found that they still reacted to poliomyelitis virus.

Dr. Bang commented that in his laboratory they had tried several experiments with the dilution phenomenon in mind. Since the amount of virus present was not known in even the titered material obtained from Dr. Murray, the infective materials were arbitrarily diluted up to 1:100. The results had been negative.

Dr. Paul asked Dr. Murray to comment on the dilution phenomenon as it occurred in the inoculation of human volunteers.

Dr. Murray said that he had conducted only one study in which the infected plasma (NIH-8) was inoculated after progressive dilution, and hepatitis developed in recipients who received material diluted to as much as 10^{-4}. Since NIH-8 was composed of infected plasma which had already been considerably diluted by addition of plasma from supposedly healthy donors, plasma from active cases of hepatitis might be expected to produce disease after even greater dilution. Studies conducted in Dr. Stokes' laboratory indicated that this was probably so, since acute phase whole blood diluted to 10^{-6} still produced disease when inoculated.

Dr. Werner Henle added that this whole blood diluted to 10^{-6} was injected in 50 cc. quantities, so that each cubic centimeter of the original blood contained at least 20,000 infectious doses.

The dilution factor would seem of great importance in situations where restricted numbers of cells were available, as in tissue cultures.

Dr. MacCallum asked whether the tissue culture workers were quite satisfied that in the initial washing of the primary explant cells they were getting rid of all the antibodies which might be present.

Dr. Syverton answered that last year Dr. Sabin, too, had raised the question of inadequate washing away of antibodies, because of his experience with pseudorabies and vaccinia. In Dr. Syverton's laboratory attempts had been made to quantitate the persistance of antibody after washing, and there was no evidence to indicate that after three washes there was any significant holdover of antibody. Both high-titer monkey serum and human serum had been used in these washing studies.

Dr. Gertrude Henle said that experience in their laboratory with poliomyelitis virus indicated that the serum antibody levels could be so high that even after washings, sufficient antibody remained within the tissue culture to inhibit the multiplication of viruses.

Dr. Bang added that he was about to conduct additional studies of the effects of small serum antibody residua on viruses in tissue cultures. In attempts to grow hepatitis virus in HeLa cell cultures, he had grown the cells in horse serum for several weeks before inoculation, so it was reasonably certain that antibodies against human hepatitis had not persisted.

Dr. Paul then asked Drs. Werner Henle and Dalldorf to present their reports on attempts to propagate the hepatitis virus in chick embryos.

B. Chick Embryos

REVIEW OF ATTEMPTS AT ADAPTATION OF HEPATITIS VIRUS TO THE CHICK EMBRYO

DR. WERNER HENLE

Considerable efforts have been made over the past decade to adapt the viruses of infectious and serum hepatitis (IH and SH, respectively) to the chick embryo. Many of these attempts appeared to be clearly unsuccessful (2, 16, 17, 19, 22) and such failures rarely are published (3). In some instances, however, the results indicated the passage of an agent (5-9, 13, 14, 20, 23, 24), yet in no case could it be proved beyond question that either IH or SH viruses had been transferred to the chick embryo.

The sources of virus for these studies were presumed or known infectious materials derived from patients at various stages of either infectious or serum hepatitis and collected as early as the pre-icteric phase or as late as 60 days after onset of illness. The materials chosen included duodenal secretions (1, 5-9, 15, 20-24), blood and blood products (2, 7-11, 13, 14, 16, 19, 20, 22, 23), feces (10, 13, 22), bile (7-9, 22), urine (5, 6, 10, 22), nasal washings (3, 10, 16), liver biopsies (1), and sternal aspirations (20, 22); or liver (3, 16, 22), spleen (16, 22) and mesenteric lymph nodes (22) obtained at autopsy. Some of these types of materials were used more frequently than others. These were inoculated into chick embryos at various stages of development (after 6 to 13 days of primary incubation) depending upon the route to be used. Most frequently, the materials were inoculated onto the chorio-allantoic membrane (1, 3, 5, 6, 10, 16, 19, 20, 23, 24) or into the amniotic cavity (3, 11, 13, 14, 22), although injections were made into the allantois (3, 7-9, 19, 20, 22), the yolk sac (11, 19, 22), veins (3), and even into the air space (21). In most instances the embryos survived and no significant lesions were observed (1, 2, 10, 11, 12-15, 16, 17, 19, 22). In others, death of the embryos resulted from the injections (5-9, 20, 21, 23, 24), or particulate components interpreted as elementary bodies were seen in materials derived from the inoculated eggs (1, 7-9).

A lethal effect of such inocula was reported by German workers (5-9, 21, 23, 23), particularly when filtrates of duodenal secretions derived from hepatitis patients served as the source of virus for passage series on the chorio-allantoic membrane or in the allantois. Similar specimens obtained from patients with other diseases were said to be lethal less frequently or not at all. The lethal factor could be transmitted through several passages, but as a rule was lost upon the fourth or fifth transfer or, in exceptional cases, by the eighth or ninth. Others (15) could not confirm these results, in that similar death rates were noted on first passage when duodenal secretions of patients with hepatitis and other diseases were used and few or no deaths occurred on further transfers. The lethal effect maintained for a few passages was taken by some authors as proof of transmission of hepatitis viruses to chick embryos.

No efforts have been made to determine whether this effect could be neutralized specifically by convalescent sera.

In other studies a search was made for elementary bodies. Benda *et al.* (1) observed particulate components in impression smears of allantoic membranes of chick embryos inoculated with liver suspension, duodenal juice, or chorio-allantoic membrane emulsions derived from five consecutive passages. These were not seen after inoculation of salt solutions. Such controls were insufficient to exclude the possible transfer of particulate host components, which subsequently were taken to be elementary bodies. Essen and Lembke (7-9) examined their inocula, as well as the allantoic fluids collected from the injected eggs, by means of the electron microscope and demonstrated the presence of spherical particles of a high degree of density to the electron beam, with an average diameter of about 180 mμ. These components, which they refer to as elementary bodies, were seen in filtrates of duodenal secretions of patients with infectious as well as serum hepatitis, but not with other illnesses, except for one patient with Weil's disease. They also were seen in the allantoic fluids of the first few passages, but apparently did not increase in number; on the contrary, they seemed to diminish in number upon consecutive passages. They were not noted after heating of the preparations to 59° C. The same type of particle was also observed to be abundantly present in filtrates of 0.25 per cent fecal suspensions. But taking the data supplied by the authors on "elementary body" counts, it would appear that between 1 and 10 per cent of the stools consisted of such particles. These various considerations militate against acceptance of the interpretation made by the authors. Here again, appropriate neutralization tests could have provided the answer. Furthermore, if the particles actually represented hepatitis viruses, their apparent concentrations in some of the preparations would appear to be sufficient to permit their identification by complement fixation techniques.

In the absence of recognizable effects following inoculation of infectious or passage materials the serologic approach to the detection of viral propagation might afford some hope of success, but thus far no positive results have been reported, and other tests for the presence of virus are needed. In a number of instances volunteers were given passage materials, with variable results. MacCallum and Bauer (19) attempted propagation of SH virus on the chorio-allantoic membrane or by the allantoic or yolk sac routes, and materials from the fourth, sixth, and seventh passages were administered subcutaneously and intranasally to volunteers without results. Similar experiments with chick embryo tissue culture materials had indicated that the virus had been carried at least through seven transfers (10^{-7} dilution of the icterogenic serum used for initiation of the passage series), but fifteenth-passage material (10^{-11} of the original seed) failed to induce illness in two subjects. Similarly, Kilham and Murray (17) failed to demonstrate virus in chick embryo passage materials. A few volunteers fed by Gordon (11) with egg materials derived from the fifteenth yolk sac passage of IH virus

developed some evidence of disease. However, the illness was not characteristic, and further inoculation experiments with yolk sac or amniotic passage materials failed to induce disease. Essen and Lembke (7-9) inoculated volunteers by the intravenous route and by nasal spray with allantoic fluids containing "elementary bodies." Of seven subjects thus exposed, five developed signs of hepatitis in 11 to 30 days with enlargement of the liver, positive Takata reactions, and increased urobilinogen in the urine. One of these patients became slightly jaundiced on the thirty-seventh day, with a serum bilirubin level of 1.83 mg. per cent. On the whole, the disease was mild. However, these results were obtained with allantoic fluids of the third or fourth passages, and thus the possibility of a carry-over of the original seed virus has not been excluded.

In the author's laboratory, several passage series of IH virus were carried out initially in chick embryo or rabbit liver tissue culture and continued later in the amnion of chick embryos (13), or were initiated directly in the amnion (18). Materials obtained at various passage levels were fed to volunteers. The results showed that agents which had been carried in series through more than 15 passages were capable of inducing a disease in some of the subjects after an average incubation period of 24 days. The clinical and laboratory findings were compatible with mild non-icteric hepatitis (4). A similar though milder disease was seen in volunteers inoculated by Leftwich and Mirick following inoculation of egg-passage materials furnished by G. Henle (18). An amniotic passage series of SH virus likewise indicated that an agent had been carried through 15 transfers, in that some of the volunteers injected subcutaneously with amniotic fluid developed an illness compatible with anicteric hepatitis after incubation periods in excess of 60 days. Sera obtained during the acute stages from volunteers exposed to materials from the IH and SH passage series, respectively, were transmitted to other volunteers with similar results except that the diseases induced appeared to be somewhat more severe. However, convalescents from the SH culture disease failed to resist challenge with "unadapted" virus of the same strain which had been used for initiation of the passage series, and jaundice developed in some of the volunteers. Similar challenge experiments with volunteers after recovery from the IH culture disease indicated immunity in an earlier test (13), but no resistance in a later study carried out by Leftwich and Mirick (18).

These results seem to indicate, then, that some agents were present in the egg materials, but they do not furnish proof that these were, or were not, IH or SH viruses. In the analysis of our experimental data the following points, among others, must be considered (18):

1. **Pick-up of a virus from the chick embryo.**—This seems unlikely in view of the fact that the incubation periods in volunteers were short when passage materials presumably containing IH virus were fed and that they were long when SH lines were injected.

2. **Excessive challenge in the immunity studies.**—Unfortunately, no

titrated natural viruses were available for challenges of the volunteers convalescent from the egg virus diseases. It is possible that the doses used were excessive and capable of overcoming any degree of immunity that might have been present.

3. **Too-early challenge.**—It is conceivable that solid immunity might develop rather late, particularly in view of the fact that in some of the volunteers infected with the egg viruses, abnormal liver function tests were noted over periods of several weeks. However, the shortest intervals between recovery and challenge were approximately two months, and some immunity could reasonably have been expected at that stage.

4. **Rapid attenuation of hepatitis viruses in the chick embryo.**—This would not be without precedent. Certainly the observations made by Rake and his associates (25, 26) with measles virus, passed in the chick embryo, indicated rapid attenuation of this agent in that after only five to seven passages the disease induced in children was mild, and that with later passages only minor evidence of infection could be produced. The immunity to subsequent exposures was far from solid. Observations with egg-adapted mumps virus (Enders *et al.* (27), G. Henle and her associates (28, 29)) indicated a similar course of events.

5. **The possibility of a dual infection in hepatitis.**—If hepatitis in man represents a dual infection, as in mice (Gledhill and Andrewes (30)), one may consider that only one of the agents has been passed in the chick embryo. However, immunity to one agent presumably should protect against the full-blown disease.

Amniotic fluids of the IH passage series were also employed as skin-test antigens, with results which were initially encouraging (12). The data obtained, often under "blind" test conditions, seemed to correlate well with the past experience of the subjects tested: known convalescents from IH gave a high incidence of positive reactions, whereas few such reactions were noted in young children and in random sampling of adults, and tests in convalescents from SH gave intermediary values. However, in later tests in epidemic areas such correlations were not regularly obtained, and nonspecific reactions with the control antigen on occasion confused the picture. It must be assumed now that either the early results represented chance correlations, or some non-specific factor has appeared in the later preparations which was not present initially. Since, in spite of many efforts, it has not been possible to clarify this situation, further work on this antigen has been abandoned for the time being.

This brief review will have amply substantiated the introductory remarks that thus far adaptation of hepatitis viruses to the chick embryo has not been proved. In any event, unless it can be established that the lethal effects observed by some workers in chick embryos are due to hepatitis virus, the egg-adapted agents, whatever they are, have not supplied the laboratory tool so urgently needed. As long as there are no serologic tests available, the question of the exact nature of the agents

passed in the chick embryo cannot be answered. If and when such tests become available, it is hoped that the answer to the nature of the egg viruses may be obtained in retrospect with the aid of the preserved sera collected from the volunteers used in these studies.

References

1. Benda, L., Gerlach, F., Rissel, E., and Thaler, H.: Über untersuchungen zur frage der frage der virusätiologie der hepatitis, Arch. ges. Virusforsch. *4*:2, 89-117, 1949.
2. Cameron, J. D. S.: Quart. J. Med. *36*:139, 1953.
3. Colbert, J. W., Jr.: Review of animal experimentation in infectious hepatitis and in serum hepatitis, Yale J. Biol. and Med. *21*:335-343, March 1949.
4. Drake, M. E., Kitts, A. W., Blanchard, M. C., Farquhar, J. D., Stokes, J., Jr., and Henle, W.: Studies on agent of infectious hepatitis; disease produced in human volunteers by agent cultivated in tissue culture or embryonated hen's egg, J. Exp. Med. *92*:283-297, Sept. 1950.
5. Dresel, E. G. and Weineck, E.: Über das virus der hepatitis epidemica, Ztschr. f. Immunitätsforsch. u. exper. Therap. *106*:21-38, 1949.
6. Dresel, E. G., Meding, G., and Weineck, E.: Über das virus der hepatitis epidemica, Ztschr. f. Immunitätsforsch. u. exper. Therap. *103*:129, 10 June 1943.
7. Essen, K. W. and Lembke, A.: Kann der erreger der hepatitis epidemica als identifiziert gelten, Klin. Wchnschr. *27*:749-753, 15 Nov. 1949.
8. Essen, K. W. and Lembke, A.: Zur ätiologie der hepatitis epidemica, Ztschr. klin. Med. *145*:517-533, 1949.
9. Essen, K. W. and Lembke, A.: Über der erreger der hepatitis epidemica, Zentralbl. Bakt. *159*:387-394, June 1953.
10. Findlay, G. M., MacCallum, F. O., and Murgatroyd, F.: Observations bearing on the aetiology of infective hepatitis, Tr. Roy. Soc. Trop. Med. & Hyg. *32*:575-586, Feb. 1939.
11. Gordon, I.: Unpublished data.
12. Henle, G., Drake, M. E., Henle, W.. Stokes, J., Jr.: Skin test for infectious hepatitis, Proc. Soc. Exper. Biol. & Med. *73*:603-605, April 1950.
13. Henle, W., Harris, S., Henle, G., Harris, T. N., Drake, M. E., Mangold, F., and Stokes, J., Jr.: Studies on agent of infectious hepatitis; propagation of agent in tissue culture and embryonated hen's egg J. Exper. Med. *92*:271-281, Sept. 1950.
14. Henle, W., Drake, M. E., Henle, G., and Stokes, J., Jr.: Evidence for the propagation of the virus of serum hepatitis in the chick embryo, Arch. ges. Virusforsch. *4*:5, 612-620, 1952.
15. Herzberg, K.: Über tragungsversuche au Kanarienvögeln mit hepatitis-contagiosa-material, Zentralbl. Bakt. *151*:81, 28 Jan. 1944.
16. Hoyle, L.: Note on some unsuccessful attempts to demonstrate virus in infective hepatitis, Monthly Bull. Min. of Hlth. and Emerg. Publ. Hlth. Lab. Serv. *2*:99-101, Oct. 1943.
17. Kilham, L. and Murray, R.: Unpublished.
18. Leftwich, C. I., Mirick, G. S., and Henle, G.: To be published.
19. MacCallum, F. O. and Bauer, D. J.: Homologous serum jaundice: transmission experiments with human volunteers, Lancet *1*:622-627, 13 May 1944.
20. Oliver-Pascual, E., Sanz-Ibanez, J., Castillo, E., Galan, J., Oliver, A., and Hernandez, M.: Contribuciones etiológicas y clínicas al problema de las hepatitis agudas infecciosas; las hepatitis epidémicas, como hepatoenteropatia, Rev. espan. enferm. ap. digest. *5*:573-626, Sept.-Oct. 1946.

21. Pendl, O.: Zur ätiologie der hepatitis contagiosa, Ztschr. klin. Med. *2*:789-820. Sept. 1947.
22. Sawyer, W. A., Meyer, K. F., Eaton, M. D., Bauer, J. H., Putnam, P., and Schwentker, F. F.: Jaundice in Army personnel in Western region of United States and its relation to vaccination against yellow fever, Am. J. Hyg. *40*:35-107, July 1944.
23. Siede, W. and Luz, K.: Zur ätiologie der hepatitis epidemica; weitere unters uchungen zum virusnachweis, Klin. Wchnschr. *22*:70, 23 Jan. 1943.
24. Siede, W. and Meding, G.: Zur ätiologie der hepatitis epidemica, Klin. Wchnschr. *20*:1065-1067, 25 Oct. 1941.
25. Shaffer, M. F., Rake, G., Stokes, J., Jr., and O'Neill, G. C.: Studies on measles; experimental disease in man and monkey, J. Immunol. *41*:241-257, June 1941.
26. Maris, E. P., Rake, G., Stokes, J., Jr., Shaffer, M. F., and O'Neill, G. C.: Studies on measles; results of chance and planned exposure to unmodified measles virus in children previously inoculated with egg passage measles virus, J. Pediat. *22*:17-29, Jan. 1943.
27. Enders, J. F., Levens, J. H., Stokes, J., Jr., Maris, E. P., and Berenberg, W.: Attenuation of virulence with retention of antigenicity of mumps virus after passage in embryonated egg, J. Immunol. *54*:283-291, Nov. 1946.
28. Henle, G., Stokes, J., Jr., Burgoon, J. S., Bashe, W. J. Jr., Burgoon, C. F., and Henle, W.: Studies on prevention of mumps; effect of oral spraying of attenuated active virus, J. Immunol. *66*:579-594, May 1951.
29. Bashe, W. J., Jr., Gotlieb, T., Henle, G., and Henle, W.: Studies on the prevention of mumps. VI. The relationship of neutralizing antibodies to the determination of susceptibility and to the evaluation of immunization procedures, J. Immunol. *71*:76-85, 1953.
30. Gledhill, A. W., Dick, G. W. A., and Andrewes, C. H.: Production of hepatitis in mice by the combined action of two filterable agents, Lancet *2*:11, 509-511, 13 Sept. 1952.

REPORT ON ATTEMPTS TO PROPAGATE THE AGENT OF HUMAN HEPATITIS IN THE CHICK EMBRYO

DR. GILBERT DALLDORF

We followed Dr. Henle's lead, and from the fall of 1951 until June, 1953, made repeated efforts to demonstrate infectious hepatitis virus in chick embryo specimens by feeding them to human volunteers. Dr. Irving Gordon was in charge of the work, but since he cannot be here today I will summarize his experiments.

He began with serum from a patient with moderately severe icteric hepatitis, one of the cases present in an outbreak in a state institution. The serum was inoculated directly into eggs and the embryos, five to six days old when inoculated, were incubated for 10 days more; the tissues, and often the embryos, were then harvested and inoculated into other eggs. Material from the fifteenth passage was fed to 10 volunteers. Three complained of fatigue and minor aches and pains, and were believed to have enlarged livers. None became jaundiced, and liver function tests yielded normal values. It is doubtful that these three were infected with hepatitis virus, since pooled serum from two of them failed to infect 10 other volunteers, and since some months later Dr. Gordon

fed material from a fresh passage of the same line to an additional group of volunteers without effect.

Dr. Henle had used the amniotic route, so the same starting material was also inoculated in this way. The thirtieth generation was fed to volunteers and they remained well. Two and one-half months later four of these volunteers were fed Akiba serum. The Akiba serum pool was known to be infectious. Two developed frank hepatitis after 32 and 35 days. The challenge dose had been a 1:100 dilution of serum. Dr. Gordon had shown that a 1:1000 dilution caused hepatitis in but one of five volunteers and a 1:10,000 dilution caused no disease whatever. His challenge dose had therefore been reasonable, and presumably neither the amniotic nor the yolk sac line had been infectious.

Feces and serum from two of the three volunteers who succumbed to challenge were used as starting material for additional transfers by both amniotic and yolk sac routes, and the fifth generation fed to volunteers in large amounts as mixtures of various suspensions. One group of 11 received as much as 100 ml. of mixed specimens, while seven others were given a third as much. None became ill.

Finally, the Akiba strain serum that had been used for challenge was itself inoculated into eggs via the yolk sac and the fifth generation fed to volunteers. These too remained well.

It seems reasonable to conclude that infectious hepatitis virus was not cultivated by the methods used from the specimens tested. We have every intention of continuing this effort by all the means we have available, because hepatitis is a serious problem and lies in an area of medicine which should be emphasized by public health practitioners.

DISCUSSION

Dr. Paul then commented that in September, 1953, at the International Congress of Microbiologists held in Rome, a report from one German laboratory had claimed successful propagation of hepatitis virus in chick embryos, with later transmission of the egg-propagated disease to humans, and had quoted several other German laboratories as similarly successful.* Dr. MacCallum would discuss this report in his review of recent experimental work in Europe.

Dr. MacCallum said that it sufficed for the present to say that this required confirmation by other workers.

Dr. Murray stressed the importance of achieving a proper understanding of serum hepatitis in man before using human transmission experiments as proof of laboratory propagation. After his experience with cases of experimental human hepatitis, he had become more cognizant of the possible fallacy in designating some illnesses as "anicteric cases." In its usual form, hepatitis is very definite and often dramatic

---

* Essen, K. W.: Neue experimentelle und Klinische untersuchungen über das Hepatitis-virus, Riassunti delle Communicazioni, VI Congresso Internazionale de Microbiologia, 2:33 (Rome) 1953.

in onset, and unless all of the clinical and laboratory findings occur in proper sequence, it is often difficult to be sure that the diagnosis is correct.

Some interesting data on challenge had also evolved from the experiments with volunteers. Eighteen volunteers who had previously been inoculated with infected materials had now been challenged with the same material. Most of these men had become ill with hepatitis after the original inoculation. In no cases did abnormal clinical or laboratory findings develop after the second challenge. These studies were so integrated with other studies in progress that controls were available for most of the materials which were used for challenge, and these were shown to be highly infective. It would seem that in these 18 cases, at least, a firm homologous immunity existed after recovery from a case of homologous serum hepatitis.

Dr. Stokes asked if the men of this group who had been ill, actually had homologous serum hepatitis, and whether they were each infected with a single virus strain.

Dr. Murray answered that the patients were assumed to have had homologous serum hepatitis. A few had illness with short incubation periods, but it had been difficult to determine whether these represented infectious or homologous serum hepatitis, since the average incubation period was about 45 days. Some had been inoculated with a single strain derived from known carriers, although others received pooled material derived from several patients.

Dr. Bang emphasized the need for defining clearly what the inoculation into man of materials from normal chick embryos would do. It seemed that even the first injection of such materials could produce some illness in man, and to evaluate transmission experiments involving materials grown in chick embryos, the effects of the embryonic constituents themselves must be known.

Dr. Werner Henle recalled that he had inoculated volunteers with normal amniotic materials and had noted no abnormalities during a three-week follow-up period.

Dr. Syverton asked whether the normal amniotic material had consisted of pooled fresh material or of substances obtained from eggs after serial passage.

Dr. Werner Henle replied that it was pooled fresh material.

Dr. Evans asked whether any of the individuals skin-tested by Dr. Henle with normal amniotic fluid became ill.

Dr. Werner Henle said that there was some evidence that intradermal injection of normal irradiated material produced minor abnormalities in tests of hepatic function in a few subjects.

In one early series it seemed that skin testing with infected, irradiated material had conferred immunity, since no cases of hepatitis developed in individuals skin-tested with infected materials during a training school epidemic, whereas many cases developed in those who

received control material. Because of later uncertainties as to the validity of the skin test, however, it was abandoned by the Pennsylvania group. Recent preliminary reports from Dr. Katz, in Chile, suggested that the egg-prepared skin-test antigen sent to him was producing reliable results, with no more than 5 to 10 per cent positive reactions among the controls.

Dr. Evans added that the experience he had had with skin reactions to material obtained from eggs after serial passage of infectious material also suggested that non-specific reactions were elicited by egg derivatives. Initially, 20 of 24 individuals with hepatitis had reacted positively to supposedly infected sera passed through six tissue cultures and seven egg passages, whereas only one of 46 hepatitis patients tested with control materials reacted positively. But nine of 10 "normal" patients (i.e., patients on the orthopedic wards) reacted positively to amniotic fluid suspected of containing hepatitis virus. Since influenza virus in the amniotic fluid was not inactivated by the General Electric bulb originally used as an ultraviolet source, a more potent quartz lamp of German manufacture was used for the irradiation of amniotic fluid given to the "normal" patients, and the egg proteins may have been altered in some way. Later experience with materials subjected to varying numbers of tissue culture and egg passages showed that the reactions to both control and hepatitis materials were of equivalent magnitude, both in percentage and in terms of individual response, so that it was never possible to tell whether reactions were due to the passaged infectious materials or to the egg or tissue culture vehicle.

Dr. Paul then requested that Drs. Mirick, Morris, and MacCallum present their papers on attempts to propagate hepatitis virus in rodents.

C. Rodents

REVIEW OF ATTEMPTS TO PROPAGATE THE VIRUS OF HUMAN HEPATITIS IN RODENTS

DR. GEORGE S. MIRICK

Viral hepatitis in man has not yet been adapted unequivocally to a rodent, despite numerous attempts. In 1948 Colbert (1) reviewed the published reports and much unpublished work up to that time. Guinea pigs, hamsters, jerboas, rabbits, and various strains of mice and rats had yielded negative results. The most promising report was by MacCallum and Miles (2). Employing Wistar rats, which were fed a protein deficient diet for seven to 40 days and then inoculated with human infectious hepatitis virus, they had apparently established a fatal, transmissible disease characterized by inflammation and necrosis of the liver and lesions in lymph nodes, stomach, intestines, and lung. The survival period was 11 to 50 days. The agent was filtered through a gradocol membrane A P D 63 and titered 1/5000. The disease appeared in the third blind passage and was carried through nine serial passages. A

neutralization test with sera from patients convalescent from hepatitis was promising. Unfortunately the agent was lost, and repeated attempts to repeat the experiments by the original authors (3) and by others (4) have failed.

No noteworthy reports of hepatitis in rodents other than mice have appeared since Colbert's review. In mice, 12 reported attempts to establish hepatitis were negative. Since then it has become apparent that at least four types of hepatitis are indigenous to mice. For convenience these will be designated types I-IV and described separately.

Type I. In 1932, Findlay (5) described acidophilic intranuclear inclusions occurring in 0.2 to 4.16 per cent of the hepatic nuclei of nearly all mice of the Clacton strain. Seven other strains of mice were examined and found free of the inclusions. No evidence of disease was present in the affected mice. The agent could be passed to uninfected strains of mice, and was thought to be a virus of low pathogenicity. Similar inclusions were subsequently described in other strains of mice by Thompson (6), Nicoleu and Ruge (7), Olitzky and Casals (8), and Pavilanis and Lépine (9), Olitzky pointed out that the lesions first appeared in Swiss or Rockefeller Institute mice at four months, and increased in number with ageing. Pavilanis and Lépine noted jaundice in two mice with these inclusions and transmitted the inclusions through three passages in mice and to guinea pigs without further evidence of disease.

Type II. In 1951, Jordan and Mirick (10), while attempting to adapt human hepatitis virus to O'Grady strain (Bagg) mice, described a disease characterized by ascites and hepatitis occurring three to four weeks after the intraperitoneal inoculation of mouse organ brei in serial passage. The ascites hepatitis agent (AHA) could not be filtered, was inactivated by heating at 56° C. for 30 minutes, and had low virulence (mortality was of the order of 2 per cent). It could not be identified with any agent known to infect mice. No inclusion bodies were seen. No immunity resulted from infection, and the agent could not be neutralized. It could be transmitted only by injection. All strains and ages of mice tested were equally susceptible. The infected livers were two to four times normal size, and showed marked infiltration with mononuclear cells and occasional areas of central and diffuse necrosis. The parenteral inoculation of volunteers with this agent in mouse organ brei resulted in an illness thought to be a foreign protein reaction but not typical hepatitis. Subsequently Lackey, Eichman, and Havens (11) and Morris (12) have isolated a similar agent in mice. The former workers found that both CFW and Bagg strains of albino mice carried the agent, and that four inbred strains were susceptible. Morris has identified acidophilic intranuclear and intracytoplasmic inclusions, probably of protozoan origin, in some of his mice, and feels that immunity to infection with the acites-producing agent does develop.

Type III. In 1951, Gledhill and Andrewes (13) described a very different disease occurring in S strain mice and virulent especially for young VS mice. This disease has a short incubation period, five to eight

days, and is highly lethal for susceptible suckling mice. The livers showed diffuse parenchymal necrosis. The disease was inhibited by aureomycin and terramycin, which conferred resistance to inoculated mice. Subsequently (14, 15) it has been shown that the disease is a complex infection due to two agents acting in concert. MHV, the "mouse hepatitis virus" causing the disease, consists of a mixture of the L (labile) component, which is indistinguishable from Eperythrozoön coccoides, and the S (stable) component, which is apparently a filterable virus. The S component, which is present in higher titer, may be obtained in pure form by passage at limiting dilutions, by treatment with terramycin, or by allowing the mixture to stand for 24 hours at 20° C. Either component may be passed in series singly without producing disease, but together they produce fatal hepatitis in susceptible weanling mice. Immunity follows recovery, and neutralization may be demonstrated. Healthy carriers have been found among strain S mice.

Type IV. In 1952, Nelson (16) described a somewhat similar disease, which also carries a form of lukemia, in weanling mice of the Princeton strain; 98 per cent of these mice died 4.3 to 6.6 days following parenteral inoculation with infectious liver extract. They had focal and diffuse necrosis of the liver cells, and the occasional surviver developed cirrhosis. The agent was filterable through a Berkfeld V candle, titered 10^{-7}, and was quite stable. Aureomycin and terramycin had no inhibiting effect. No neutralizing antibody was found in convalescence, and no chronic carriers of the agent were demonstrated. The remarkable differences between strains of mice in susceptibility to this agent is noteworthy. Weanling mice of Swiss, NSVS, C Albino, and Bagg strains showed mortalities of 4, 12, 20, and 0 per cent in older mice of that strain. This disease resembles MHV infection but differs in lack of immunity, smaller size and higher titer of the agent, and resistance to antibodies. The agent might represent a more virulent form of the S component of MHV.

There are some recent reports in the Japanese literature which are difficult to assay because of insufficient information. Kimura and Hotta (17) in Kyoto have described the adaptation of human hepatitis virus to mice by intrahepatic inoculation of presumably infective blood (IH). Through 15 serial passages, 20 to 80 per cent of inoculated mice died four to nine days after inoculation; their livers were diseased and showed infiltration and necrosis. The intracutaneous inoculation of one volunteer with 0.4 ml. of mouse brei from the seventh passage was said to result after nine days in non-icteric hepatitis. No liver function tests were performed. One wonders if this was not a foreign protein reaction.

Hara and Kashiwagi (18) in Osaka have described the isolation of three strains of hepatitis virus in mice. The first strain was thought to be derived from the liver of a patient who died on the forty-first day of disease with acute yellow atrophy of the liver. Eight mice weighing 6 to 10 gm. died 12 to 21 days after the intraperitoneal inoculation of 0.2 ml. of the liver suspension. During 60 subsequent serial intraperitoneal passages of infected organ extracts, all mice died in five to 10

days. The livers showed extensive infiltration and necrosis. The agent was filtered through a Berkfeld candle and titered 10^{-8}. With the electron microscope uniform particles 100-150 mμ were seen which were not present in control material. A neutralization test with human convalescent hepatitis serum showed "remarkably diminished virulence" of the virus at a dilution of 10^{-6}. The other two strains of virus these workers describe were thought to be derived from the blood obtained early from patients with hepatitis. They were thought to be identical to the first strain.

A soluble antigen was prepared from the spleens of mice infected with the first strain and inactivated by heating at 80° and 70° C., respectively, for one hour on each of two days. This antigen, together with a control prepared from normal mouse spleens, was tested intradermally and read at intervals for 36 hours. A positive skin test developed in all of 31 patients with hepatitis during the third or fourth week of disease. The test was positive in 80 per cent of 67 patients with a history of previous hepatitis, in 41 per cent of 71 patients with liver disease other than hepatitis, and in 16 per cent of 77 patients with no history of hepatitis or evidence of liver disease.

The meaning of these findings is not clear, but the many nonspecific immune reactions, to be described by Dr. Havens, which occur in patients with hepatitis demand their cautious interpretation. The agents described by the Japanese workers seem to have many characteristics of the mouse viruses described by Andrewes *et al.* and by Nelson.

It is clear that there are several agents, in addition to LCM and ectromelia virus, which are indigenous to mice and may cause hepatitis, hepatic necrosis, and death. Any claim to the adaptation of human hepatitis virus to this species must be supported by unequivocal evidence such as the retransmission of typical disease to humans or clear-cut neutralization or complement-fixation tests with human sera.

References

1. Colbert, J. W., Jr.; Review of animal experimentation in infectious hepatitis and serum hepatitis, Yale J. Biol. and Med. *21*:335-343, March 1949.
2. MacCallum, F. O. and Miles, J. A. R.: Transmissible disease in rats inoculated with material from cases of infective hepatitis, Lancet *1*:3-4, 5 Jan. 1946.
3. MacCallum, F. O.: Personal communication.
4. Ratnoff, O. D. and Mirick, G. S.: Unpublished experiments.
5. Findlay, G. M.: Intranuclear bodies in liver-cells of mice, J. Exper. Path. *13*:223, June 1932.
6. Thompson, J.: Am. J. Path. *10*:376, 1934.
7. Nicoleu, S., and Ruge, H.: Arch. ges. Virusforsch. *3*:260, 1944.
8. Olitzky, P. K., and Casals, J.: Certain affections of liver that arise spontaneously in so-called normal stock albino mice, Proc. Soc. Exper. Biol. & Med. *60*:48-51, Oct. 1945.
9. Pavilanis, V., and Lépine, P.: Ann. Inst. Pasteur *76*:280, 1949.
10. Jordan, J. and Mirick, G. S.: Hepatitis in mice of presumed viral origin, Bull. Johns Hopkins Hosp. *89*:328-331, Oct. 1951.

11. Lackey, M. D., Eichman, P. L., and Havens, W. P., Jr.: Hepatitis in laboratory mice, J. Inf. Dis. *93*:1, 14-20, July-Aug. 1953.
12. Morris, A.: Personal Communication.
13. Gledhill, A. W. and Andrewes, C. H.: A hepatitis virus of mice, Brit. J. Exper. Path. *32*:559-568, Dec. 1951.
14. Gledhill, A. W., Dick, G. W. A., and Andrewes, C. H.: Production of hepatitis in mice by the combined action of two filterable agents, Lancet *2*:11, 509-511, 13 Sept. 1952.
15. Niven, J. S. F., Gledhill, A. W., Dick, G. W. A., and Andrewes, C. H.: Further light on mouse hepatitis, Lancet *2*:22, 1061, 29 Nov. 1952.
16. Nelson, J. B.: Acute hepatitis associated with mouse leukemia. I. Pathological features and transmission of the diseases, J. Exper. Med. *96*:293-302, Oct. 1952.
17. Kimura, R. and Hotta, S.: Experimental studies of infectious hepatitis; transmission of a viral agent from human hepatitis into livers of white mice, Acta Scholae med. Univ. Kioto *30*:2, 133-139, 1952.
18. Hara, K., Kashiwagi, B., *et al.*: Proc. Japan Acad. *28*:270, 1952; *28*:511, 1952; *29*:83, 1953.

ATTEMPTS MADE AT THE ARMY MEDICAL SERVICE GRADUATE SCHOOL TO PROPAGATE THE AGENT OF HUMAN HEPATITIS IN RODENTS

DR. J. ANTHONY MORRIS

Efforts at the Army Medical Service Graduate School to propagate the viruses of human hepatitis in an experimental host have frequently been complicated by the occurrence of latent agents of infectious disease in apparently healthy laboratory animals. This was especially so in mice when it was necessary to resort to serial tissue passage. In one instance, clinical materials from a fatal case of serum hepatitis were inoculated into Bagg mice obtained from Mrs. Flora O'Grady. Suspensions of tissues of the inoculated mice were passed in series to other mice obtained from the same commercial source. In one of the passage lines a transmissible illness characterized by ascites and hepatosplenomegaly occurred after a few passages. To determine the significance of these results, serial mouse tissue passages were carried out in normal mice of the same strain, and transmissible ascitic disease was again encountered. By means of serial passage, an agent resembling a protozoan and apparently capable of producing ascites and hepatosplenomegaly was encountered in tissues of apparently healthy mice. This agent was indistinguishable from the agent recovered in mice originally inoculated with human material which, pending identification, is hereinafter designated MAA (mouse ascites agents).

Mice inoculated with suspensions of tissues infected with MAA appear to remain well for 12 to 15 days, after which they become less active and develop abdominal swelling. Practically all sick mice recover after two to four weeks of illness. Mice killed three to four weeks after inoculation of infectious materials show enlarged, pulpy livers, splenomegaly, and usually 5 to 10 cc. of ascitic fluid. Microscopically,

the hepatic lesions are characterized by focal infiltration of mononuclear cells and by necrosis of parenchymal cells (figure 1).

Figure 2 illustrates the protozan-like structures seen in an impression smear from the surface of the spleen of a mouse sacrificed 21 days after inoculation with MAA agent. The Giemsa-stained intracytoplasmic structures are blue and surrounded by a clear zone; when stained by Machievello's method, such structures are red.

The ascites-producing agent resists exposure to 56° C. for 30 minutes and to —70° C. for prolonged periods, but is inactivated by lyophilization. It is completely sedimented by centrifugation at 3,000 rpm for 30 minutes and is held back by a Seitz EK filter pad. Its virulence for mice is low and has not increased as passages were continued. Young adult guinea pigs, hamsters, rabbits, rhesus monkeys, and embryonated eggs are uniformly resistant. However, moderate ascites was present in one of two albino rats inoculated intraperitoneally with early mouse passage material, but attempts to repeat these results with early or late passage materials have failed.

Thus, it is seen that the disease induced in mice by MAA and the ascitic diseases encountered in mice by Jordan and Mirick (1) and by Lackey and her co-workers (2) are similar in many respects, and that the etiologic agents of the three diseases share many characteristics. Accordingly, efforts were made to determine whether the disease in our mice and that described by Jordan and Mirick have a common etiology. The agent of the latter disease, ascites hepatitis agent (AHA), was received at the Army Medical Service Graduate School in March 1952 in the fifteenth mouse passage, and has been maintained by serial passage in Bagg and Ajax mice. Efforts were made to find the protozoan-like bodies associated with MAA in stained impression films of tissues of mice infected with AHA. These were found in mice of the twentieth passage, but it is not possible to assess the significance of their presence since it is now established that apparently healthy Bagg mice harbor latent MAA infections. Nevertheless, cross-resistance tests were performed in mice with MAA and the Army Medical Service Graduate School strain of AHA. Groups of mice convalescent from MAA and our strain of AHA, as well as mice convalescent from scrub typhus, were inoculated with a suspension of mouse tissues infected with MAA. Other mice convalescent from MAA and AHA were inoculated with a 10 per cent suspension of mouse tissue infected with AHA. Table I summarizes the findings. It is seen that mice convalescent from MAA and our strain of AHA were resistant to reinfection with MAA, whereas control mice and those convalescent from scrub typhus were susceptible. Conversely, mice convalescent from MAA and our strain of AHA resisted challenge with AHA, while control mice did not.

Encephalitozoön infections, which have been encountered in our laboratory in mice employed in isolation studies concerned with hemorrhagic fever on at least a half dozen occasions during the last several years, may induce in mice a disease similar in many respects to the ascitic

TABLE I

CROSS-RESISTANCE TESTS BETWEEN MAA AND AHA ASCITIC AGENTS

Challenged with	Convalescent from	Number mice dead or with ascites 21 days after challenge with dilutions of			
		10^{-1}	10^{-2}	10^{-3}	10^{-4}
MAA	Controls	6/6	6/6	3/6	0/6
	MAA	1/9			
	AHA	0/9			
	Scrub Typhus (Karp strain)	16/16			
AHA	Controls	8/8	8/8	2/7	1/8
	MAA	0/36			
	AHA	0/29			

diseases just discussed. In fact, there is as yet no completely satisfactory method of differentiating MAA from Encephalitozoön. However, the most constant and characteristic histologic lesion induced in mice by Encephalitozoön is a meningoencephalitis (3). This lesion was not present in any of an appreciable number of mice infected with

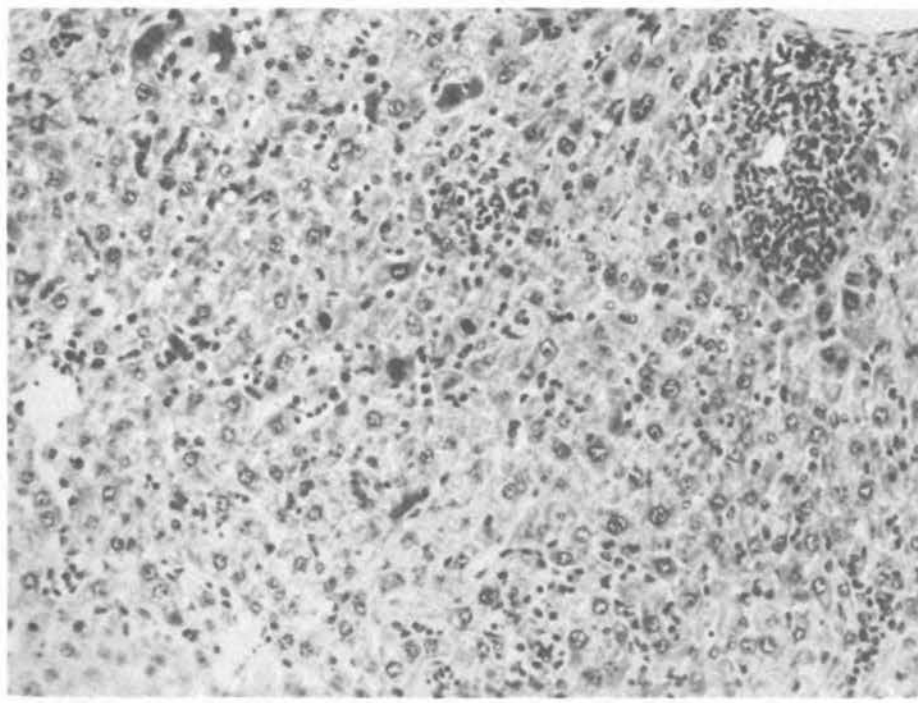


Fig. 1. Photomicrograph of the liver of a mouse sacrificed 30 days after infection, illustrating scattered infiltrations of mononuclear cells and an occasional parenchymal cell undergoing necrobiotic change. (X150)

MAA. Furthermore, the grapelike clusters of parasites commonly present in the kidneys of mice infected with Encephalitozoön are not seen in the kidneys of mice infected with MAA.

In connection with the mouse hepatitis virus (MHV) of Gledhill and Andrewes, it may be of interest to give a brief account of studies carried out in our laboratory concerned with MHV and other viruses antigenically related to it. A recently isolated virus (H747), which was recovered in suckling mice in Japan, consistently produces a fatal disseminated encephalitis in mice one to ten days old following intracerebral inoculation of appreciable quantities of infectious material. By means of neutralization and complement-fixation procedures, the H747 virus was shown to be antigenically related to MHV as well as to two other mouse viruses, JHM (4) and EHF120 (5). The viruses comprising this antigenically related group, which has been designated the murine hepatoencephalitis group of viruses, had not previously been shown to belong to the same immunologic family. However, Gledhill and his co-workers demonstrated some antigenic relationship between JHM and MHV, but the exact degree of relationship could not be determined, since the neutralization values of the sera prepared against the viruses were low and the titration results were uneven.

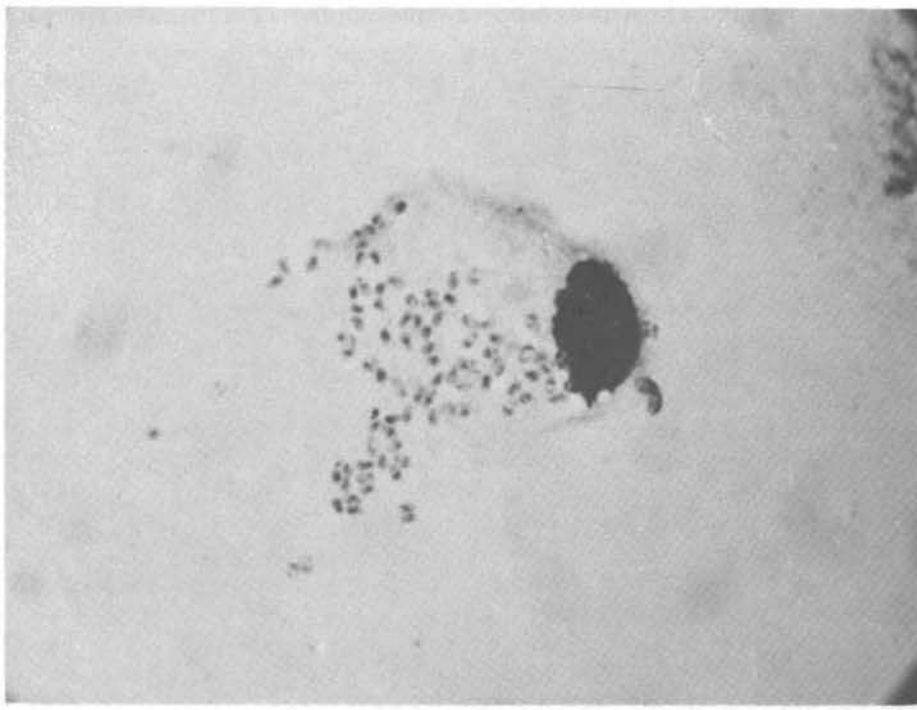


Fig. 2. Photomicrograph of impression smear of surface of the spleen of a mouse sacrificed 21 days after experimental infection with ascites producing agent showing the protozoan-like structures in a disrupted cell. (X1200)

It remains to be determined whether the HEV virus recovered in mice in Australia by Stanley and his colleagues (6) is antigenically related to the murine hepatoencephalitis group of viruses.

In summary, it is worth reiterating that a number of enzoötic hepatic diseases are encountered in laboratory mice, and that these diseases may confuse the worker who attempts to propagate the viruses of human hepatitis in this host.

References

1. Jordan, J. and Mirick, G. S.: Hepatitis in mice of presumed viral origin, Bull. Johns Hopkins Hosp. *89*: 328-331, Oct. 1951.
2. Lackey, M. D., Eichman, P. L., and Havens, W. P., Jr.: Hepatitis in laboratory mice, J. Inf. Dis. *93*:1, 14-20, July-Aug. 1953.
3. Perrin, T. L.: Arch. Path. *36*:559-567, Dec. 1943.
4. Gledhill, A. W. and Andrewes, C. H.: A hepatitis virus of mice, British J. of Exper. Path, *32*:559-568, Dec. 1951. (Quoted by Cheever, F. S., Daniels, J. B., Pappenheimer, A. M., and Bailey, O. T.: A murine virus (JHM) causing disseminated encephalomyelitis with extensive destruction of myelin, J. Exper. Med. *90*:181-194, Sept. 1949.)
5. Annual Report, 406th Medical General Laboratory, 1952.
6. Stanley *et al.*: Austral. Jour. Biol. and Med. *31*:147, April 1953.

REPORT ON STUDIES OF VIRUS HEPATITIS IN MICE

G. W. A. Dick, A. W. Gledhill and Janet S. F. Niven

National Institute for Medical Research
Mill Hill, London

Presented by Dr. F. O. MacCallum

Mouse hepatitis virus (MHV) produces non-fatal hepatitis in just-weaned VSBS mice, but a fatal disease results in these mice when they are simultaneously infected with Eperythrozoön coccoides. MHV by itself produces fatal hepatitis in infant mice one to ten days of age, and in them E. coccoides makes no difference to the outcome. Further studies of MHV have shown that it is carried in a latent form by a proportion of mice of all colonies which we have examined. We have no evidence that MHV naturally causes hepatitis in VSBS mice.

We have (a) confirmed that MHV inoculated intraperitoneally into just-weaned mice protects them against challenge with a mixture of MHV and E. coccoides, and have shown that this protection is in evidence when the challenge is made at intervals as short as 12 hours, and (b) shown that mice which naturally carry MHV are susceptible to challenge with MHV and E. coccoides. This is concluded from observations on the susceptibility and virus carriage rates of comparable groups of mice. In order to explain how MHV rapidly induces resistance to challenge with a mixture of MHV and E. coccoides, we have made the hypothesis that the majority of MHV particles infecting liver cells do not forthwith multiply and that cells thus infected are resistant to further infection with MHV, just as lysogenic strains of bacteria are

rendered resistant by prophage contained within them. The same hypothesis would explain the susceptibility of carrier mice if we assume that only a small proportion of their liver cells contain virus, the remainder being fully susceptible. The persistence of infection of carriers we attribute to the occasional development and liberation of MHV from cells containing it. We are exploring methods of causing the liberation of virus from infected cells and thereby attempting to substantiate the hypothesis. We have also kept in mind that our experimental series of suckling and just-weaned mice are in a rapid growth with varying virus susceptibility.

Mouse hepatitis resembles homologous serum jaundice (SH) in having no known natural history, and in becoming evident on inoculation of homologous materials. The discovery of the natural mode of transmission of MHV might throw some light on that of SH jaundice. Furthermore, the hypothesis mentioned above regarding MHV would carry several possibilities if applied to SH jaundice. Firstly, the active agent (or even substance) in infected serum could cause hepatitis by liberating hepatitis virus in a carrier, just as homologous liver tissue containing E. coccoides produces severe or fatal hepatitis in a MHV carrier. Alternatively, human carriers of hepatitis virus might be resistant to challenge with infected sera because the majority of their liver cells were refractory to infection by reason of virus already present in them. As with MHV, resistance would not depend upon the presence of demonstrable antibody.

In addition to MHV, we have isolated what we believe is a variant of it. This was recovered from mice which had been inoculated with acute phase serum from a patient (Craig). Craig virus differs from MHV in that it is fatal for just-weaned mice at high dilutions in the absence of E. coccoides. Its properties are otherwise very similar to those of MHV. We have also produced neurotropic variants of MHV, of Craig virus, of the mouse hepatitis virus isolated by Nelson, and of a hepatotropic variant of JHM virus. These four viruses are in many respects alike and have been shown to be antigenically related.

MHV produces not only liver damage, but also an unusual histological reaction characterized by widespread focal formation of multinucleated giant cells from the mesothelium of both thorax and abdomen, and from the endothelium of venous channels of all orders of size. This vascular change is most striking, the multinucleate masses projecting into the lumen; although so widespread, it is of transient duration, the syncytial masses disappearing quickly with rapid restoration of endothelial continuity. It is of interest that although E. coccoides plays no part in the reaction, the giant cells are more numerous with the double infection where the MHV titer is relatively higher. In the cytoplasm of affected liver cells a bizarre accumulation of basophilic material precedes cell death, and on many occasions these inclusions have first been found in close relation to giant cells arising from the endothelium of hepatic veins.

Craig virus and MHV are both neutralized by human gamma globulin. Since there is no evidence that either of them is related to human hepatitis we have not further explored this neutralization by gamma globulin. (If it is specific, this neutralization would be explained if these viruses were related to some obscure human infective agent.)

We have applied our knowledge of MHV in attempts to adapt infectious hepatitis (IH) and SH viruses to small laboratory animals. Simultaneous inoculations of infective sera and E. coccoides, Plasmodium berghei, Babesia rodhaini, or Haemobartonella muris have not resulted in adaptation of the viruses; nor have attempts to combine infective sera and MHV been successful. We have also studied the effect of combined inoculations of rats with H. muris and IH and SH sera, but we have failed to relate the hepatitis which we have produced to the use of infected sera and have not pursued this further.

We hope to publish shortly a series of papers giving the results of our experiments to date.

DISCUSSION

Dr. MacCallum stated that in his work with Dr. Miles on hepatitis in rats, they had found that the diet of a rat could be so balanced as to produce liver lesions similar to acute hepatic necrosis in man; this animal also provided a suitable host for studying the effect of infectious agents producing toxins which ordinarily would be removed by the liver. The mouse viruses did not appear to be infectious for rats, nor were laboratory-bred rats so prone to severe bacterial infections as mice. Most strains of laboratory rats were, as was generally known, carriers of bartonnella organisms, whose presence became evident when the spleen was removed. So far, there had been little evidence of a latent hepatitis virus in rats, although the extremely stable rat virus isolated by Novy in 1909, and used again by Jordan and his colleagues in 1952, immediately brought to mind the stability of the serum hepatitis agent in man. Reyniers and his colleagues at Notre Dame had also recovered an agent in their germ-free young rats which had the characteristics of a virus. This produced a disease which manifested symptoms indicative of involvement of the central nervous system and which could be transmitted to germ-free rats, but not to normal rats.

Dr. Smadel mentioned that the recent Japanese claims of positive passage of human hepatitis to mice, which Dr. Mirick had described, should be considered in the light of some of Dr. Morris' recent findings. When Dr. Morris was working on epidemic hemorrhagic fever last spring in the Far East, he discussed this agent with Dr. M. Kitaoka, of the National Institute of Health in Tokyo, who had also studied the mouse "hepatitis" strain. Dr. Kitaoka expressed the opinion that it was the same mouse virus which had been isolated a few years previously during the early studies on hemorrhagic fever, and which was designated EHF-120. As Dr. Morris had pointed out, the EHF-120 strain was antigenically related to MHV, JHM, and to H-747. H-747 had been isolated the preceding year during studies on epidemic hemorrhagic fever, was

neurotropic, and provided clean-cut neutralization tests. Dr. Smadel indicated that his group had encountered this mouse contaminant so often in Japanese mice that the members were inclined to minimize its importance as an agent of human disease. At any rate, they had not actually studied the samples for which claims were made.

Dr. Dalldorf added that his group had a virus which had recently been isolated at Bar Harbor from a tumor strain of mice and which was very similar to the JHM agent. He had never been able to pick up such agents in his own mouse colonies, however.

He asked whether Dr. Cheever had not once believed that his JHM virus was of human origin.

Dr. Enders answered that Dr. Cheever did not feel that the JHM virus was derived from man. The agent was obtained from a paralyzed mouse which had not been inoculated.

Dr. Paul asked Dr. Mirick if it would be possible to differentiate the mouse viruses from the human hepatitis virus by determining their resistance to heating for 30 minutes at 60° C.

Dr. Mirick replied that resistance to heat might well be a possible differential point, though he did not have information as to the resistance of the Japanese hepatitis agent to heat.

Dr. Murray recalled that hepatitis still occurred in human volunteers inoculated with samples of the NIH infected plasma pool which had been heated for four hours at 60° C.

Dr. Werner Henle commented that heating might be unreliable as a differential test, since a few virus particles resistant to heat might survive and be sufficient to produce disease.

Dr. Enders asked if all of the antigenically related groups of viruses described by Dr. Morris produced demyelinization, as did JHM.

Dr. Morris replied that H-747 would kill only suckling mice, which possessed insufficient myelin to show clear-cut changes; JHM, on the other hand, produced death in mice of 8 to 10 grams, and myelin changes were pronounced.

Dr. Dalldorf stated that the differential susceptibility with age was interesting, but might not be paramount. The strain with which he had recently been working had initially produced disease only in young mice, but older rodents had recently been found susceptible, also.

Dr. Mirick asked Dr. Morris whether he had studied the effects of antibiotics on Mouse Ascites Agent (MAA).

Dr. Morris replied that the agent resisted penicillin, streptomycin, aureomycin, and terramycin, and, although it seemed to be protozoan, also resisted antimalarial compounds.

Dr. Smadel asked Dr. MacCallum whether the successful neutralization tests with immune mouse serum reported in the paper of Drs. Dick, Gledhill, and Nevins had been undertaken with some of the earlier variants of mouse hepatitis virus, before a lethal neurotropic strain

was obtained. At the Army Medical Service Graduate School, it had been difficult to obtain clean-cut neutralization tests until the neurotropic H-747 strain turned up.

Dr. MacCallum answered that the neutralizations were performed with the recently-adapted variants of MHV and of Craig virus.

Dr. Evans then summarized the results of attempts to produce hepatitis in mice which he had carried out in Germany and which Dr. Robert McCollum had carried out at Yale. A total of 28 mice were given urethane and then inoculated with materials (stools, sera or plasma, bile, or liver from patients with viral hepatitis), which were believed to contain the agent of SH or IH. Nineteen of the 28 materials produced ascites in mice, more commonly on the second or third passage than on the first passage. The ascites produced was as irregular in its appearance, however, as it was in Dr. Mirick's experience. Twenty-four control mice were given urethane and then inoculated with several materials which did not contain virus (saline, stools from normal individuals, serum from normal individuals and from patients with obstructive jaundice, nomal bile, and normal human liver). Eight of the 24 control materials produced ascites. The incidence of ascites in mice receiving suposedly infected materials was, therefore, 86 per cent, and in those receiving control materials, 33 per cent. The following table summarizes the results:

ATTEMPTS TO PRODUCE HEPATITIS IN MICE

	McCollum[1] no. pos./no. inoc	Evans[2] no. pos./no. inoc	Total no. pos./no. inoc	Total Per cent Pos.
Hepatitis Inocula				
Stool	3/3	2/2	5/5	100
Sera or Plasma	8/10	0/1	8/11	73
Bile	1/1		1/1	100
Liver		5/5	5/5	100
Hepatitis Total	12/14	7/8	19/22	86
Control Inocula				
Saline or Nothing	0/2	1/2	1/4	25
Stools (normal)	1/2		1/2	50
Sera (normal)	2/4	2/7	4/11	36
Obstructive Icterus	2/5		2/5	40
Bile (normal)	0/1		0/1	0
Liver (normal)		0/1	0/1	0
Control Total	5/14	3/10	8/24	33

(1) McCollum, R. O.: (Unpublished experiments) Preliminary report in Ann. Rep. (1951-52) Commission on Virus and Rickettsial Diseases, AFEB, Submitted to Office of the Surgeon General, Department of the Army, April 1952.

(2) Evans, A. S.: Unpublished experiments carried out in 1951-52 at Hepatitis Research Center, 98th U. S. Army General Hospital, Germany.

Despite the somewhat higher incidence with the infected materials, the disease appeared in such an irregular fashion that the "agent" could not

be titered or studied with Seitz filtration or heat inactivation. Confusingly, ascites sometimes appeared when mice had received liver biopsy material from a patient with hepatitis which was mixed with convalescent serum from the same donor patient.

Dr. Paul asked if Dr. Evans felt that the difference between the 86 and 33 per cent incidence was significant.

Dr. Evans replied that he did not. Not as many passages had been carried out with the control material as with the infected material, and that may have made the study unbalanced.

Dr. Smadel asked whether the studies using control and infected material had been conducted simultaneously. He had noted in his own laboratory, in the course of hemorrhagic fever studies, that agents which had appeared only once during a six-to-nine-month period of serial blind passages in mice could appear three to four times during a later two-month period of similar passages.

Dr. Evans answered that controls were run simultaneously with test materials in some experiments, but separately in others, and that Dr. Smadel had certainly pointed out an additional fault of the study.

Dr. Sabin added that he would like to add an experience from his own laboratory to the accumulated reports of unsuccessful attempts at propagating human hepatitis in rodents. One of his associates had contracted a febrile illness while he was working with dengue and sandfly fever, and samples of his blood were obtained 12 hours after the onset of the disease. Dr. Sabin decided to utilize his 12-hour serum in the same procedures which had proved successful in adapting dengue and sandfly fever viruses to rodents. Accordingly, the serum was inoculated into two series of newborn mice, and blind passages were carried out at 14-day intervals, on the assumption that a long incubation period might be present. One series of passages were done intracerebrally with brain material, and the other intraperitoneally with liver and spleen material. However, after three blind passages no disease was obtained in either series.

Dr. Paul then asked Dr. Syverton to describe his recent attempts to transmit infectious hepatitis and serum hepatitis to antibody-free newborn swine.

D. SWINE

EXPERIMENTS ON THE TRANSMISSION OF INFECTIOUS HEPATITIS TO ANTIBODY-FREE NEWBORN SWINE *

DRS. JEROME T. SYVERTON, GEORGE A. YOUNG AND K. THEODOR BRUNNER

A variety of materials from patients ill with hepatitis have been employed for transfer to swine in attempts to induce experimental hepatitis (1-4, 6, 9). The test materials have included blood, duodenal fluid,

* Aided by grants from the National Institutes of Health, Public Health Service; The Lederle Laboratories Division, American Cyanamide Co., and the Hormel Foundation, Austin, Minnesota.

stomach washings, bile, urine, feces, vomitus, and liver and other tissues in suspension. Recipient pigs differing in age, nutritional status, and physical well-being were employed in attempts to obtain a host of decreased resistance. Despite this evidence for the insusceptibility of swine to infection by hepatitis viruses (1-4, 6, 9), swine deserve renewed investigation since this species resembles man more closely anatomically and physiologically than most experimental animals. It is the purpose of this report to describe briefly the results to date of pilot studies designed to test the susceptibility of newborn, antibody-free miniature swine to infection by the viruses of infectious hepatitis (IH) and serum hepatitis (SH).

MATERIALS AND METHODS

Animal host.—Sixteen miniature swine were made available by one of us (G.A.Y.) for these experimental studies. These baby swine were a by-product of a genetic experiment made under the direction of Dr. David C. England, Hormel Institute, University of Minnesota, for the production of a small breed of swine, which at six months of age, ranged in weight from 25 to 50 lbs. and in height from 12 to 16″ (10, 11). A sample of the type and growth characteristics of the baby swine employed in this study is illustrated by figure 1 (page 54). To eliminate the possibility that the test animals might acquire antibodies for the hepatitis viruses or for other agents, by passive transfer or by infection at birth or

TABLE I

RESULTS OF EXPERIMENTS SH-1, SH-2, SH-3 AND SH-4
Attempts to Infect Baby Swine with Serum Hepatitis Virus (SHV)

		Test Serum		Swine Recipients				Interpretation
Exp. No.	Strain	Amount in ml.	Route	Pig No.	Age in Days	Fever	Days Observed	Production of Hepatitis
SH-1	NIH-8 (Murray)	2	I.P.	SH-1	11	+*	52	Not proved
	L-181 (Murray)	2	I.P.	SH-2	11	0	126	Not proved
	L-204 (Murray)	2	I.P.	SH-3	11	0	126	Not proved
SH-2	NIH-8 (Murray)	0.5	I.P.	SH-4	9	0	142	Not proved
	L-204 (Murray)	0.5	I.P.	SH-5	9	0	128	Not proved
SH-3	L-181 (Murray) L-204 L-207	2	I.P.	SH-6	19	0	23	Not proved
	NIH-8 Merriam	2	P.O.					
SH-4	NIH-8 (Murray)	0.8	I.P.	SH-7	−2	0	23	Not proved
	L-204	0.3	I.N.					
	L-204 (Murray)	0.3	I.N.	SH-8	−2	0	30+	Not proved
	L-204 (Murray)	0.3	I.N.	SH-9	−4	0	30+	Not proved
	L-204 (Murray)	0.3	I.N.	SH-10	−4	0	30+	Not proved

* Death 11/21/53 from Escherichia coli septicemia.

TABLE II

Results of Experiments IH-1, IH-2, IH-3
Attempts to Infect Baby Swine with Infectious Hepatitis Virus (IHV)

Test Material								Swine Recipients					Interpretation
Exp. No.	Date	Type	Strain	Source	Inoculum	Amt. in ml.	Route	Pig No.	Age in Days	Fever	Other Symptoms	Period of Observation	Production of Hepatitis
IH-1	1/29/54	IH	H-19	A.L. fatal human case	Spleen Liver	2.0 2.0	I.P. P.O.	IH-1	13	0	0	23	Not proved
	1/29/54	IH	M-2000 M-2001 M-2002 M-2003	Pool from 4 patients	Fecal	1.5 0.5	I.P. P.O.	IH-2	19	0	0	23	Not proved
IH-2	1/29/54	IH	"	"	Fecal	1.5 0.5	I.P. P.O.	IH-3	19	0	0	23	Not proved
	3/1/54	IH	H-19 H-20 M-2001 M-2002 M-2003	5 patients with I.H.	Pool of Liver Spleen Feces Feces	2.0 0.3	I.P. I.N.	IH-4	—2	0	0	30+	Not proved
IH-3	3/1/54	IH	"	"		2.0 3.0	I.P. I.N.	IH-5	—4	0	0	30+	Not proved

shortly thereafter, the baby pigs were delivered by Caesarean section and each pig was transferred without benefit of colostrum or sow's milk to a single isolation unit where it was kept from extraneous infectious agents. The isolation units were modified from the Horsfall and Bauer unit (5) for maintenance of pigs (12). The diet from birth consisted of modified cow's milk fed thrice daily from a shallow flat pan within the unit. These miniature pigs can be kept successfully in such units for weeks. Electrophoretic studies established the absence of gamma globulin in the sera of control animals. Pigs delivered prematurely were used for the final experiments in each series and for experiments underway.

Test materials for inoculation.—The materials used for inoculation have been described (7). The data that relate to these test materials are contained in tables I and II. The preparation of contaminated materials for inoculation was carried out as described elsewhere for the recovery of virus from material containing a mixed microbial flora (8).

RESULTS

Antibody-free, baby miniature swine were tested for susceptibility to the viruses of hepatitis. The specimens employed were described fully in an accompanying report from this laboratory (8) which concerns the use of human epithelial cells, strain HeLa, for the study of hepatitis.

Experiments SH-1, SH-2, SH-3, and SH-4 were carried out in attempts to infect baby swine with the agent of serum hepatitis. Five specimens of human serum, each presumed on good evidence to contain the agent of serum hepatitis, were employed singly and as pools for transfer by intraperitoneal injection, by intranasal instillation, and/or by mouth feeding to ten pigs ranging in age from at least four days premature to 19 days. During the observation period, which was from 23 to 142 days, these animals were protected from extraneous infection and from adverse environmental changes by continuous incubation in single isolation units. The data and results for these experiments are contained in table I. The death of pig SH-1 in experiment SH-1 from Escherichia coli septicemia resulted shortly after removal from the isolation unit. The temperature of these animals ranged from 99.8° to 104.4° F. with total irregularity from day to day and without apparent relationship to infection. The continuous gluttonous appetite shown by each pig was accepted as reasonable evidence for lack of illness. The findings recorded in table I were interpreted as evidence of failure to transmit the agent of serum hepatitis to antibody-free baby swine.

Essentially similar experiments were carried out employing specimens representative of infectious hepatitis (table II). These specimens had been obtained from patients ill with the clinical disease, but the infectivity of the specimens had been established by transfer to man.

Table II, representative of the results of experiments IH-1, IH-2, and IH-3, reflects tests that were carried out with materials from six patients. These materials consisted of suspensions of liver, spleen, and feces. Test materials were used singly or as pools, as indicated. The

routes of transfer were intraperitoneal, oral and intranasal. The five recipient baby pigs were observed for from 23 to 30 days, and showed no evidence suggestive of hepatitis. The baby pigs delivered at least two and four days prematurely and infected immediately following delivery by Caesarean section continue to be under observation. The findings recorded in table II were interpreted as evidence of failure to transmit the agent of infectious hepatitis to antibody-free baby swine.

SUMMARY

Baby pigs delivered by Caesarean section and transferred without benefit of colostrum or sow's milk to isolation units were employed in attempts to transmit the agents of serum hepatitis and of infectious hepatitis. These antibody-free baby swine were kept in isolation under observation for from 23 to 142 days from the day of inoculation without any evidence suggestive of clinical hepatitis.

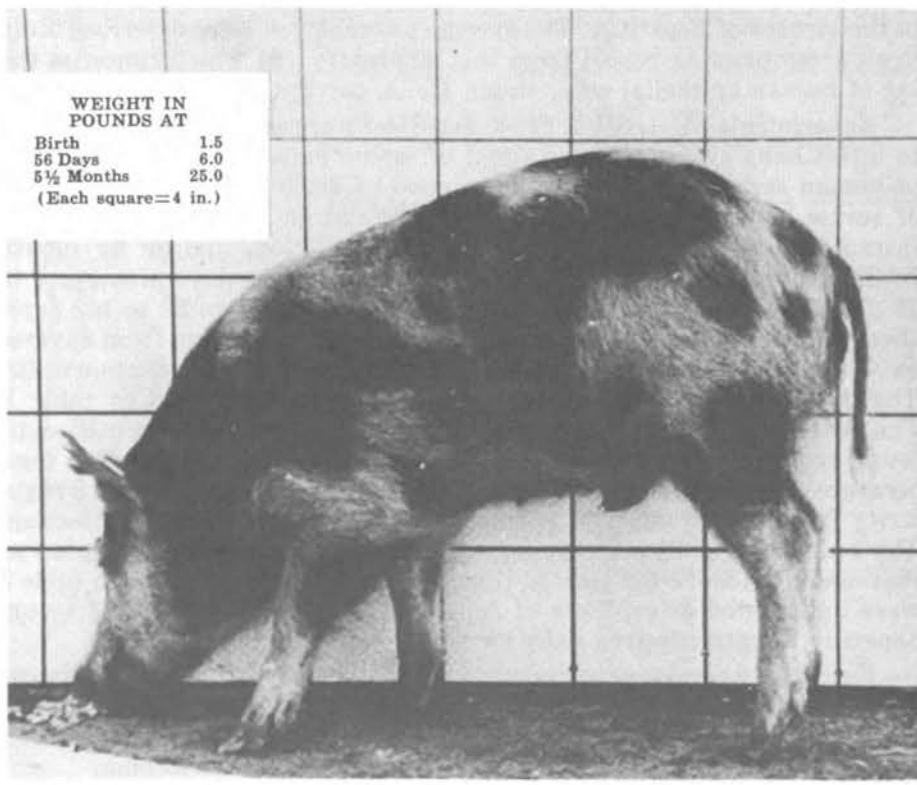


FIGURE 1. Miniature pig at five and one-half months to illustrate the experimental host employed for this study. This new and useful experimental animal at maturity is about a tenth the size of the average commercial hog.

References

1. Andersen, T. T., and Tulinius, S.: The etiology of hepatitis epidemics, Acta. med. Scandinav. *95*:497, 1938.
2. Colbert, J. W., Jr.: Review of animal experimentation in infectious hepatitis and in serum hepatitis, Yale J. Biol. and Med. *21*:335-343, March 1949.
3. Findlay, G. M., and MacCallum, F. O.: Hepatitis and jaundice associated with immunization against certain virus diseases, Tr. Roy. Soc. Trop. Med. & Hyg. *31*:799, 1938.
4. Findlay, G. M., MacCallum, F. O., and Murgatroyd, F.: Observations bearing on the aetiology of infective hepatitis, Tr. Roy. Soc. Trop. Med. & Hyg., *32*:575-586, Feb. 1939.
5. Horsfall, F. L., Jr., and Bauer, J. H.: Individual isolation of infected animals in a single room, J. Bact. *40*:569-580, 1940.
6. Sergiev, E. G., Tareev, E. M., Gontaeva, A. A., Livschiz, I. M., Sanvinskii, G. N., Trofimovski, N. A., and Zimmerman, A. N.: Virus jaundice—epidemic hepatitis in relation to immunization with human serum, Terap. Ark. *18*:595, 1940. (Quoted by Colbert, J. W., Jr., Yale J. Biol. and Med. *21*:335-343, 1949.)
7. Syverton, J. T., Scherer, W. F., and Elwood, P. M.: Studies on the propagation *in vitro* of poliomyelitis viruses. V. The application of strain HeLa human epithelial cells for isolation and typing, J. Lab. & Clin. Med. *43*:286-320, 1954.
8. Syverton, J. T.: Serum hepatitis and infectious hepatitis: the use of human cell cultures, HeLa strain, this Symposium.
9. Van Rooyen, C. E., and Gordon, I.: Some experimental work on infective hepatitis in the Middle East forces, J. Roy. Army Med. Corps, *79*:213, 1942.
10. England, D. C., Winters, L. M., and Carpenter, L. E.: The development of a miniature breed of swine—a preliminary report, Journal of Animal Science, *12*:4, 894 (an abstract) 1953.
11. England, D. C.: The development of a new experimental animal for medical research: The miniature pig, Proc. 4th Annual Meeting Animal Care Panel, University of Chicago, 1953. In press.
12. Young, G. A., and Underdahl, N. R.: Isolation units for growing baby pigs without colostrum, Am. J. Vet. Res. *14*:571, 1953.

DISCUSSION

Dr. Evans commented that experiments had been carried out at the Hepatitis Research Center in Munich with newborn suckling pigs. However, these pigs had certainly not been as carefully prepared as Dr. Syverton's animals, and it had not been possible to transmit human hepatitis to them.

Dr. Syverton noted that suckling pigs received maternal antibodies routinely, under natural conditions, via the colostrum in milk. Since it was presumed that hepatitis might be a disease acquired immediately after birth, and because it was hoped to avoid as much as possible the presence of pre-existing antibodies, pigs delivered by Caesarian section were selected for the experiments he had just described. Dr. Young had recently observed that pigs delivered five days prematurely by Caesarian section were extremely susceptible to an agent which produced fatal diarrhea when given by the intranasal route. It was, therefore, planned to conduct a final experiment in which material containing

human hepatitis virus would be given to pigs delivered 10 to 15 days prematurely.

Dr. Stokes asked whether the antibody-free swine developed gamma globulin after inoculation with material containing hepatitis.

Dr. Syverton replied that this had not been determined, but the animals did develop gamma globulin when they were removed from the isolation unit after the completion of the experiments.

Dr. Paul asked whether the fact that the serum electrophoretic patterns of Dr. Syverton's pigs showed no gamma globulin indicated that no maternal antibody had been absorbed through the placenta.

Dr. Syverton replied that this was his understanding. Like the bovine placenta, that of the pig is so constructed that no antibodies are transmitted through it from the mother. Maternal antibodies are, therefore, transmitted only in the colostrum.

Dr. Murray added that it seemed appropriate to sound a note of caution concerning the interpretation of the electrophoretic patterns of the sera of certain animals. The electrophoretic pattern of dog plasma, for example, is extremely difficult to evaluate.

Dr. Sabin recalled that dairy pathologists had fairly well correlated the presence of antibodies against various agents with the existence of certain electrophoretic patterns in bovine and pig sera. It had been reliably demonstrated that no antibodies were present in the sera of newborn calves or pigs if they did not get colostrum; the absence of antibodies was associated with an absence of gamma globulin. The animals began to develop their own antibodies four to six months after birth, and it was interesting to note that these antibodies had a pattern which differed from that of antibodies passively transferred in colostrum.

Dr. Smadel asked whether there were any small laboratory animals which were comparable to the pig and calf in that they received no antibodies across the placenta.

Dr. Shope replied that Wallenstein *et al.** had listed only horses, pigs, mules, goats, cattle, sheep, and deer as animals in which no placental antibody transmission occurs. All of the smaller animals tested had demonstrated complete transplacental antibody transmission.

Dr. Murray asked Dr. Syverton what criteria he had used for the development of hepatitis in his pigs.

Dr. Syverton answered that his clinical criteria consisted only of development of fever and loss of appetite. The livers of animals killed at the end of the observation period showed no evidence of abnormality.

Dr. Paul then asked Dr. Shope to describe his experiments with the transmission of hepatitis to pigs.

Dr. Shope reported that during the previous year he had attempted to transmit human serum hepatitis to six Yorkshire pigs obtained from the New Jersey Agricultural Experimental Station at Rutgers. The pigs

---

* American Veterinary Medical Association, 88th Annual Meeting, 105-109, 1951.

were eight weeks old, had been weaned, and had been kept in isolation during the experimental period, but not before. Three of the pigs were inoculated with Fort Bragg Serum 2 obtained from Drs. Stokes and Henle, two with NIH-8 plasma obtained from Dr. Murray, and one with a second-passage serum obtained from the first group inoculated with the Fort Bragg material. After incubation periods of from two to 11 days, all of the animals became ill with a disease characterized by fever, extreme anorexia, and emesis. Fevers were as high as 41° C. and lasted for as long as 11 days. The animals became lethargic, and one became transiently comatose. There was no jaundice. Although no blood studies were made, it was noted that the sick swine bled excessively when their tails were snipped according to the usual procedure used to obtain blood from such animals.

It had been impossible to obtain more pigs from the same source for confirmatory experiments, since a quarantine had been placed on New Jersey pigs because of an epidemic of vesicular exanthema. The experimental disease could not be reproduced in Chester White pigs obtained from Long Island, and efforts were being continued to obtain more animals from the original Rutgers source.

Dr. Bayne-Jones asked if the livers of the infected animals had been examined post mortem.

Dr. Shope answered that autopsies had been performed on all of the animals after they had completely recovered from their illnesses, and the livers appeared to be normal. Biopsies were not taken during the illnesses because it seemed at that time that the pigs were going to die.

Dr. Smadel asked whether it was possible that the second-passage material obtained from the first group which received Fort Bragg Strain virus and which was then inoculated into another pig, may have transmitted a contaminating pig virus.

Dr. Shope replied that he was sure the illness was not caused by a pig virus. He had never before seen an illness like this in pigs, and he had previously inoculated many of these animals with several other human materials. In addition, 15 to 20 uninoculated pigs from the same source had been maintained in isolation simultaneously with the experimental group, and none of these had become febrile or ill in any way.

Dr. Smadel asked whether material from the sick pigs had been inoculated into the second group of pigs subsequently studied.

Dr. Shope answered that passage of material from the sick Rutgers pigs to the animals obtained from Long Island had not produced disease.

Dr. MacCallum then asked Dr. Syverton whether the feces which he fed his animals were bacteriologically sterile.

Dr. Syverton replied that he could not be sure that the inoculum was absolutely sterile. However, it had shown no growth in tissue culture.

Dr. MacCallum stated that it might well be a mistake to use bacteriologically sterile material in attempts to produce infectious hepatitis by

the oral route. It was conceivable that the liver lesions in hepatitis represent simply a toxic change, with the virus exerting its direct pathological effects on the spleen, lymph nodes, gastrointestinal tract, and other tissues. If the liver lesion were a result of "toxic" damage, the bacterial flora in the gastrointestinal tract might be of great importance. This would be consistent with the finding, in many rat experiments, that the mortality rates produced by deficient diets are decreased when antibiotics are given to sterilize the gastrointestinal tract.

Dr. MacCallum asked Dr. Shope whether it is true that the gastrointestinal tract of the pig, more than that of any other animal, closely resembles the gastrointestinal tract of man.

Dr. Shope agreed that, although the pig's gastrointestinal tract is anatomically unlike that of man, its physiological functions are very similar. He added that the dietary habits of pigs would predispose them to infection with infectious hepatitis if the species were susceptible to that disease; apparently, however, the disease does not occur naturally in pigs. Pigs probably do not have a disease of their own which corresponds to serum hepatitis, since no liver disease had been associated with the prevalent use of routine immunization with large pools of immune sera obtained from other pigs.

Dr. Stokes considered it possible that young pigs, like children, might have a very mild illness with hepatitis, but no jaundice, and with only slight gastrointestinal symptoms.

Dr. Syverton stated that it was with this possibility in mind that he had worked with pigs delivered by Caesarian section. These animals were reared without resort to colostrum or sow's milk, and were kept in Horsfall-Bauer units. Since hepatitis in the newborn might be a mild, essentially inapparent disease, it was hoped that close observation would detect hepatitis in animals that had been inoculated with infected material during this most susceptible phase of life.

Dr. Paul next asked Dr. Evans to present his review of attempts to transmit human hepatitis to primates other than man.

E. PRIMATES

ATTEMPTS TO TRANSMIT THE VIRUS OF HUMAN HEPATITIS TO PRIMATES OTHER THAN MAN

DR. ALFRED S. EVANS

This paper will review attempts to transmit viral hepatitis to primates other than man. In doing this no general division of material into experiments with infectious hepatitis (virus A) or serum hepatitis (virus B) will be made, since in many instances the nature of the icterogenic material inoculated was unknown. Investigations prior to 1947 have been summarized by Colbert (1), and only a brief discussion of this material will be given. Subsequent experiments, some of them unpub-

lished, will be dealt with in more detail. Only the American, English, and more readily available European reports have been reviewed.

At the outset, it can be stated that there is very little evidence that primates other than man are susceptible to viral hepatitis. A summary of such experiments to 1947 (1) as given in table I, emphasizes the magnitude of such studies which were carried out in many countries by many investigators. The actual number of animals is probably greatly underestimated, since many reports did not record these data. A wide variety of monkey species were used in the total of over 133 inoculated, and over 25 baboons were studied. Occasional fever was encountered, but otherwise the results were completely negative with two possible exceptions. These are recorded in a paper by Findlay, Martin, and Mitchell (2). A monkey (Cercopithecus nictitans petaurista) was given six weekly injections of neoarsphenamine, inoculated with serum presumably containing virus B, then given three more injections of arsphenamine. The icteric index rose to 17 units. Death occurred 21 days after the serum inoculation. Acute hepatic necrosis was found at autopsy. A second monkey prepared in the same way and inoculated with a suspension of this liver sickened after an incubation period of 28 days and the icteric index rose from four to 12 units. At autopsy similar but less extensive liver necrosis was found. A control monkey given an equal number of neoarsphenamine injections alone was killed and a normal liver found. I have been unable to locate subsequent published work using this approach carried out by these or other investigators.

The published reports reviewed by Colbert (1) suggest that liver function tests were rarely carried out. Observation for clinical illness, the appearance of jaundice, temperature elevation, and alterations in leukocyte counts were the primary criteria used, and these were supported by occasional autopsy study of the liver. An exception to this is the careful study of chimpanzees carried out by Havens and Ward (3). The animals were inoculated with serum and stool specimens which had been shown in studies in human volunteers to contain virus A. Serial liver function tests, including measurement of bromsulfalein excretion, were then carried out frequently over a period of 65 to 120 days. One

TABLE I

SUMMARY FROM LITERATURE OF ATTEMPTS TO TRANSMIT VIRAL HEPATITIS TO PRIMATES: 1941-1947

Animal	Number of Animals	Results
Baboon	25+	Completely negative
Chimpanzee	6	Completely negative
Monkeys	133+	2 developed liver necrosis, a few fever alone, rest completely negative

Data based on review by Colbert (1).

of six chimpanzees inoculated died at 65 days of parasitic infection and hemorrhagic pneumonia. This animal had shown abnormal bromsulfalein excretion before and during the experiment, but the liver was normal at autopsy. A second chimpanzee died of acute asphyxia at 120 days, and a normal liver was found at autopsy. The other four chimpanzees remained healthy throughout the experiment, and all liver function tests remained normal except for the cephalin flocculation tests of two animals; these abnormalities had, however, antedated injection with icterogenic serum. These results are fully recorded in the paper of Havens and Ward.

SUBSEQUENT EXPERIMENTS

A brief survey of the literature has revealed only two published reports (4, 5) of attempts to induce hepatitis in primates other than man since 1947, but a number of unpublished experiments have been carried out in the section of Preventive Medicine, Yale University School of Medicine, under the auspices of the Commission on Virus and Rickettsial Diseases, Armed Forces Epidemiological Board. I am indebted to Dr. John R. Paul for permission to review and quote this material.

Marmosets: A large but uncertain number of the common marmoset (Callithrix jacchus) were inoculated with presumptively icterogenic materials by Dr. Horace T. Gardner in 1948-1949. Serial liver function tests were done before and after inoculation. It was noted that the alkaline phosphatase was quite high in normal marmosets. No significant alterations in liver function tests and no evidence of illness occurred following inoculation with hepatitis materials. It was concluded that this animal was not susceptible to hepatitis.

Monkeys: Several attempts have been made at Yale to infect monkeys since 1947. Most of these were made by Dr. Gardner. The following species were employed: Macaca mulatta, Cerococebus atys (sooty mangabey), Macaca cynomolgus, Cercopithecus aethiops sabeus (green monkey), and Cercopithecus aethiops pygerythrus (the vervet). The experiments were negative. Occasional abnormalities in liver function tests were observed in both normal and inoculated monkeys.

Mention only of successful transmission of presumed viral hepatitis to monkeys and chimpanzees is made in a report by Pellissier and Lumaret appearing in 1948 (4). These authors studied patients from an epidemic of apparent hepatitis in Uganda. An agent capable of producing disease in guinea pigs was isolated from the blood of three patients with hepatitis and from one patient with a febrile myocarditis. The agent could be filtered through a Seitz and Chamberland L 3 filter. Inoculated guinea pigs showed fever, congestion of liver and spleen, and hepatic involvement in pericentral lobules. The agent was said to be pathogenic for rabbits, monkeys (Cercopithecus), and chimpanzees, but no details are given. Tests to exclude leptospirosis were made, and blood cultures were sterile.

In collaboration with my wife and Viktoria Sturtz I carried out and published a series of experiments with monkeys conducted at the 98th U. S. Army General Hospital in Germany (5). The hope was to render monkeys susceptible to hepatitis by inoculation with ACTH, cortisone, or urethane. We also tried such approaches as inoculation with presumably infectious material, and then three weeks later giving convalescent serum from the same patient to produce an antigen-antibody reaction. Another approach along similar lines was to give repeated injections of liver suspension from two fatal cases of viral hepatitis, followed in ten days by pooled acute-phase hepatitis sera given intravenously. The results are summarized in table II. No monkey sickened, and only four showed slight rises in total serum bilirubin above the upper limit of normal which was taken as 0.20 mg. per cent. No elevation above 0.4 mg. per cent was observed. In the published paper it is mentioned that one monkey died of uncertain cause 205 days after inoculation (5). In later histological studies it was found that there was quite severe hepatitis with some necrosis. This was the only apparent cause of death. No alterations in liver function tests had occurred during the period of testing (103 days). This monkey had received duodenal juice and stool suspension from a case of hepatitis, type unknown, and had been given 25 mg. daily of cortisone for three days, beginning the day before inoculation with hepatitis material. Second passage of this monkey's liver was made. In the recipient, no illness, alterations in liver function tests, or histological abnormalities in liver biopsy specimens were observed in a period of approximately three months. The author's return to the United States necessitated interruption of the experiment before fulfillment of the 205-day period between inoculation and death seen in the first monkey.

We considered the possibility that hepatic involvement, even necrosis, might occur with little alteration in liver function tests. To test this a series of experiments was made with carbon tetrachloride in four monkeys (two rhesus and two cynomolgus). Increasing amounts of carbon tetrachloride from 5 to 25 cc. were administered through a gastric tube at seven-day intervals. The results are given in figure 1. Irregular increases in serum bilirubin occurred, with a peak at 48 hours after each dose. The highest observed was 1.3 mg. per cent. All increases were transient, lasting not more than a few days, and responses were unpredictable. No other abnormality in liver function tests occurred. One liver biopsy done some time later revealed no residual changes. These experiments pointed to the possibility that jaundice and alterations in liver function tests in monkeys might occur only in the face of severe liver damage; indeed, monkeys might be relatively resistant to hepatotoxic agents in general. In pursuit of this idea, some literature on yellow fever in monkeys was reviewed. Wakeman and Morrell (6) have published pertinent studies of the bromsulfalein excretion test and van den Bergh reaction. Ten rhesus monkeys infected with yellow fever had normal serum bilirubin values (up to 0.2 mg. per cent) during the

TABLE II

Recent Attempts to Produce Viral Hepatitis in Monkeys
(From Evans, Evans, and Sturtz (5))

Monkey Numbers	Hepatitis Inocula		Other Inocula				Length of Observations (days)**	Results
	Type	Route and Method	Type	Inoc. schedule*	Daily Dose (mg)	Total Dose (mg)		
CY 001 RH 2	Pooled early sera	Multiple Parenteral	Cortisone	−2 to +5	50	350	75	Rise in TSB in RH3 to 0.22 mg.% on 10th day
RH 30	Proved SH sera	IV and SQ	Cortisone	−2 to +4	25	400	141	Negative
RH 889	Duodenol juice Stool suspension	Parenteral Oral	Cortisone	−1 to +1	25	75	103	Negative***
RH 27	Liver biopsy suspension Stool suspension	Parenteral Oral	Cortisone	−1 to +1	25	75	103	Negative
RH 26	Liver biopsy suspension, then convalescent sera 3 weeks later	Parenteral	Cortisone	−1 to 0	25	50	117	Negative
RH 28 RH 29	Liver suspension of fatal cases	IP daily for 5 days	Cortisone	−2 to +14	25	400	139	Rise in TSB in RH 28 to 0.22 mg.% on 72nd day
RH 15 RH 7 CY 004	Pool of early sera Pool of early duodenal juice, both repeated once	Parenteral Gastric tube	ACTH	−2 to +19	40	840	115	Negative
RH 884	Proved SH sera	IV and SQ	Urethane	−3 to +57	500 (oral)	30500	141	Negative
RH 886 RH 887	Liver suspension of 2 fatal cases followed in 10 days by pooled acute phase sera	IP daily for 4 days IV	None				116	Rise in TSB to 0.28 and 0.32 mg.% in RH 886 on 25th and 53rd day respectively

* Minus and plus refer to days before and after inoculation of hepatitis materials respectively; 0 day is the day of inoculation.

**** Period during which liver function tests were done. Monkeys were observed clinically for 6 months.**

***** Died on 205th day after inoculation.**

IV=Intravenous; SQ=Subcutaneous; IP=Intraperitoneal; CY=Cynomolgus monkey; RH=Rhesus monkey; TSB=Total serum bilirubin; SH=Serum hepatitis. It is not known whether the other materials represented SH or IH.

first three days of fever, and one of these died on the second febrile day. Two other monkeys died with serum bilirubins under 1 mg. per cent. In 19 monkeys with serum bilirubin levels over 2.0 mg. per cent on the second to eighth day of known fever, 15 died within ten hours after the blood samples were taken. Of a total of 45 monkeys studied, only one survived. In this monkey no increase in serum bilirubin occurred except for a slight trace on the fourth day of fever. Thus the occurrence of jaundice was usually almost a terminal event, and death from hepatic necrosis could occur in its absence. A study of bromsulfalein excretion (5 mg./kg. of body weight) in six monkeys during a control study indicated almost complete clearance of the dye within ten minutes. The excretion of dye was delayed in all infected monkeys, but multiple samples at varying times were occasionally necessary to show this. In some, abnormal retention occurred before fever, but in one monkey it was delayed until the day before death. The bromsulfalein test was thus more readily altered than the serum bilirubin, but severe, usually fatal hepatic disease was again almost a necessary criterion.

Chimpanzees: I can find no published reports of attempts to infect chimpanzees with hepatitis since the report of Havens and Ward, except for brief mention in a paper (4) discussed above. Two unpublished experiments have been carried out at Yale University. In the first, which I carried out in 1950, serum was employed which had produced jaundice in all of three volunteers, with incubation periods to onset of symptoms of 53 to 75 days (7). This same serum was later successfully used in volunteer experiments by McCollum to determine the size of virus B (8) as being of the order of 26 mμ or less. Four young chimpanzees were inoculated intravenously with this serum, and it was also given intramuscularly in Freund's adjuvant. This latter procedure was employed in the hope that continued release of the virus over a long period, with the possibility of enhanced antigen-antibody reaction, might favor the production of experimental disease. Liver function tests, but not including bromsulfalein excretion, were done before and on the 28th, 55th, 62nd, 73rd, and 84th day after inoculation. Three chimpanzees developed sterile abscesses at the sites of intramuscular injection. Otherwise, no clinical illness occurred, and there was neither fever nor alteration in liver function tests.

In 1951 Dr. Robert W. McCollum repeated these experiments using the same serum mixed with serum from three other cases of unknown types of hepatitis. These were given in conjunction with cortisone administration. The details and results of this and other chimpanzee experiments are recorded in table III. Four female chimpanzees, aged two to three years, were inoculated intramuscularly and intravenously by McCollum, after they had been given 200 mg. of cortisone for the two preceding days. Following inoculation, cortisone was given for eight days, stopped for one week, and then given for seven days. The interrupted schedule was based on the observations that interruption of hormone therapy in human hepatitis predisposed to severe relapse (9).

TABLE III

ATTEMPTS TO TRANSMIT HEPATITIS TO CHIMPANZEES
(Experiments carried out in the Section of Preventive Medicine, Yale University School of Medicine)

Investigator (s)	Year	No. of Animals	Age of Animals	Inoculum	Route	Adjuvant	Observation Time in days	Result
Havens & Ward (3)	1945	2	12-15	Proved IH stool Proved IH serum	Oral P	None	90-120	Negative*
		4	2-3	Proved IH stool Proved IH serum	Oral P	None	65-120	Negative*
Evans**	1950	4	Young	Proved SH serum	IM	Freund's adjuvant	84	Negative (sterile abscesses at IM site in 3)
McCollum**	1951	4	2-3	Proved SH serum Unknown serum Unknown stool	IV IM Oral	Cortisone from −3 to +8 days. Stopped for 7 days, then given for 7 days (total 1.15 gm.)	117	Negative

* 1 died at 65th day after inoculation with hemorrhagic pneumonia, and 1 of acute asphyxia at 120 days. Both livers were normal.
** Unpublished studies.
Freund's adjuvant: Paraffin oil, "Falba", heat-killed tubercle bacillus.
P=parenteral; IM=intramuscular; IV=intravenous; IH=infectious hepatitis (virus A); SH=serum hepatitis (virus B).

The results were negative. No clinical illness, fever, or significant alteration in liver function tests or blood counts developed over a period of 117 days.

DISCUSSION

Almost universal failure has been met with by investigators seeking to induce viral hepatitis in baboons, chimpanzees, marmosets, and monkeys. Only the experiment of Findlay, Martin, and Mitchell (2) seems worth repeating. In this instance infectious material, presumably containing virus B, produced liver necrosis in two monkeys prepared by neoarsphenamine injections after an incubation period of three to four weeks. One other questionable experiment which we carried out using cortisone as a preparing agent suggested the remote possibility of an incubation period of over six months (5).

Much of the published material is subject to the criticism that the infectiousness of the inoculum was unknown and that the appearance of clinical illness, fever, or death constituted the only criteria of successful transmission. But modern liver function tests employed in more recent work in marmosets, monkeys, and chimpanzees have not been more revealing. There are data from experiments in carbon tetrachloride poisoning (10) and yellow fever infection (6) in monkeys which indicate that severe, perhaps fatal, liver derangement may be necessary before alterations in liver function tests become evident. Thus even should laboratory primates be susceptible to viral hepatitis, a marked, even fulminant, hepatitis might be needed before it could be recognized. In repeating efforts like those of Findlay *et al.* (2) modern tests of hepatic function, especially bromsulfalein excretion, are indicated, and liver biopsies should be attempted. We had some success with the latter method in experiments carried out with Col. Robert S. Nelson at the 98th U. S. Army General Hospital. A modified, shortened Vim-Silverman needle was used under ether anesthesia. Successful serial biopsies in the same monkey have been obtained in this way.

SUMMARY

1. The literature reporting attempts to transmit viral hepatitis to primates other than man has been briefly reviewed.

2. There is little evidence that such primates are susceptible to viral hepatitis.

3. One experiment in which liver necrosis was produced in two monkeys inoculated with virus B and prepared by neoarsphenamine injections deserves repetition.

4. Experience with carbon tetrachloride poisoning and yellow fever infection indicates that alteration in liver function tests in monkeys may accompany only severe or fatal hepatic involvement.

5. Future experiments with primates should utilize modern techniques of hepatic function, including bromsulfalein excretion and liver biopsies.

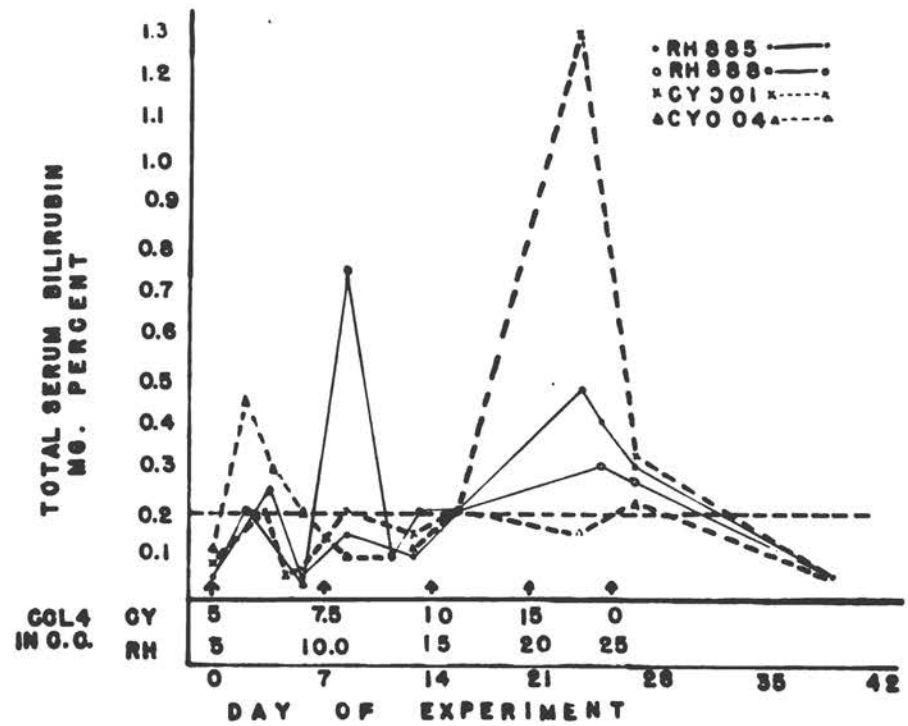


FIG. 1. Effect of oral carbon tetrachloride administration on total serum bilirubin in 4 monkeys. CY=cynomolgus monkey, RH=rhesus. Dotted horizontal line= upper level of normal.

References

1. Colbert, J. W., Jr.: Review of animal experimentation in infectious hepatitis and in serum hepatitis, Yale J. Biol. and Med. *21*:335-343, March 1949.
2. Findlay, G. M., Martin, N. H., and Mitchell, J. B.: Hepatitis after yellow fever inoculation. Relation to infective hepatitis, Lancet *2*:365-370, 16 Sept. 1944.
3. Havens, W. P., Jr., and Ward, R.: Failure to transmit infectious hepatitis to chimpanzees, Proc. Soc. Exper. Biol. & Med. *60*:102-104, Oct. 1945.
4. Pelissier, A., and Lumaret, R.: Sur un ultravirus isolé dans un foyer d'ictère epidémique sévissant en Oubangui, Ann. Inst. Pasteur, *74*:507-508, June 1948.
5. Evans, A. S., Evans, B. K., and Sturtz, V.: Standards for hepatic and hemotologic tests in monkeys: observations during experiments with hepatitis and mononucleosis, Proc. Soc. Exper. Biol. & Med. *82*:437-440, March 1953.
6. Wakeman, A. M., and Morrell, C. A.: Chemistry and metabolism in experimental yellow fever in Macacus rhesus monkeys. VI. The bromsulfalein liver function test and the van den Bergh reaction, Arch. Int. Med. *50*:876-883, 1932.
7. Evans, A. S.: Serum hepatitis in U. S. troops in Germany, Proc. Soc. Exper. Biol. & Med. *75*:809-811, Dec. 1950.
8. McCollum, R. W., Jr.: The size of serum hepatitis virus, Proc. Soc. Exper. Biol. & Med. *81*:157-160, Oct. 1952.
9. Evans, A. S., Sprinz, H., and Nelson, R. S.: Adrenal hormone therapy in viral hepatitis I, II, and III, Ann. Int. M. *38*:1115-1133; 1134-1147, 1148-1159. June 1953.
10. Evans, A. S.: Unpublished experiments carried out at the Hepatitis Research Center, 98th U. S. Army General Hospital, Munich, Germany, 1952.

DISCUSSION

Dr. Mirick described briefly some attempts which he and Dr. Ratnoff had made six years before to transmit human hepatitis to rhesus monkeys. Human serum believed to contain SH virus was the primary inoculum, and lactophenin was used as an adjuvant. Sixty monkeys were involved in the experiments, and serial passages of sera were made at six-week intervals. The monkeys were observed for one year, with weekly tests of liver function, including bromsulfalein excretions. The results were entirely negative.

Dr. Murray added that during the past year, in work done in association with Dr. Kilham, he had inoculated a number of monkeys with known infectious material. Cephalin flocculation tests on two monkeys were positive 60 to 70 days after injection. A discouraging feature of this work was the finding that normal values for tests of liver function in the monkey differ from those in man and other species. Apparently the monkey has a more rapid or efficient clearance of bilirubin from the blood, since the normal levels of serum bilirubin were lower than those in man. The cephalin flocculation test was normally negative in monkeys, although it was normally positive in most of the other animal species tested. Serum cholinesterase levels in monkeys were normally twice those encountered in man.

Dr. Murray asked Dr. Evans whether the normal monkey serum bilirubin level of 0.2 mg. per cent which he had described represented total serum bilirubin or the one-minute value.

Dr. Evans replied that this value represented total serum bilirubin, and that the normal one-minute value in monkeys was about 0.1 mg. per cent. He had determined serum bilirubin values in monkeys almost 200 times, and only in the two animals described in his report did the total values exceed 0.2 mg. per cent. This low level for normal total serum bilirubin in monkeys agreed with the levels determined by Wakeman and Morrell. The cephalin flocculation tests were invariably negative, and the thymol turbidity titers remained at normal levels except for rare slight changes which occurred during minor intercurrent infections. The bromsulfalein retention, 45 minutes after 5 mg. of dye were injected per kilo, was always under 5 per cent.

On the other hand, the serum alkaline phosphatase in monkeys and chimpanzees was often very high; normal values sometimes reached 25 or 30 Bodansky units.

Dr. Mirick noted that he had found it necessary to give monkeys 10 mg. of bromsulfalein per kilo to get more than 5 per cent retention at 25 minutes.

Dr. Evans recalled that Wakeman and Morrell had also noted this very rapid BSP excretion in monkeys. When their animals received 5 mg. of bromsulfalein per kilo the plasma was completely clear of the material in 10 minutes. Dr. Evans' group in Germany had not determined serial BSP levels, but had measured the level at 45 minutes only.

Dr. MacCallum commented that Dr. Findlay's experiments with monkeys, which Dr. Evans had described, had been conducted under difficult conditions in the tropics, and that Dr. Findlay had not attached sufficient significance to the positive results to pursue the experiments further. It would seem worthwhile to repeat on a larger scale the experiments which used arsphenamine as an adjuvant.

Dr. Smadel asked whether it was posible that the monkeys which had developed acute hepatic necrosis in Dr. Findlay's experiments had actually contracted yellow fever.

Dr. MacCallum replied that this was unikely, in view of Dr. Findlay's extensive experience with tropical diseases. However, it was essential to employ strict isolation of the experimental animals when such transmission studies were conducted in the tropics. To emphasize the importance of strict isolation, Dr. MacCallum cited an episode which had occurred in his own laboratory while he was attempting to pass human hepatitis to monkeys. Mice which had been infected with Trypanosoma cruzi in the course of another experiment were placed for a short period on shelves in the monkey room. One of the monkeys subsequently died with marked fatty changes in its liver, and when oral passage of this liver produced fevers in other monkeys, it was initially believed that hepatitis was being successfully transmitted. It was, of course, subsequently discovered that the monkey had died with a T. cruzi infection, and that it was this disease that was being transmitted by means of the liver tissue.

Dr. Paul then asked Dr. MacCallum to present his review of attempts to transmit human hepatitis to other animal hosts, and of recent European reports of successful propagation of the virus.

F. Other Animal Hosts

REVIEW OF ATTEMPTS TO TRANSMIT HEPATITIS TO OTHER ANIMAL HOSTS AND REVIEW OF RECENT EUROPEAN EFFORTS AT PROPAGATION OF THE VIRUS

DR. FRED O. MACCALLUM

The previous speakers have dealt with attempts to propagate hepatitis viruses in most of the hosts which are most suitable for laboratory experiments and which have been found satisfactory for the investigation of many viruses. However, attempts have also been made to propagate the viruses in a number of other species of animals and some birds, and even though most of these might be considered less suitable *a priori*, they would be very useful if susceptible. These animals include guinea pigs, rabbits, ferrets, dogs, cats, swine, horses, lambs, domestic fowl, and canaries. Most of these hosts have been used in work on both infectious hepatitis (virus A) and serum hepatitis (virus B). In several instances the only records of the results of the experiments

appear in a table under the heading of "negative or questionable results" in a summary by Colbert (1949). Although the results have generally been described as negative, the available information will be discussed for each of the two viruses.

My impression, in the light of our present knowledge and ideas, is that one of the most significant points about much of the past work on attempted transmission of these viruses is that the specimens used were unlikely to have contained the virus in a sufficient quantity or in a form likely to have produced the disease even in a susceptible host. Liver, spleen, and lymph glands, if not obtained from the few rapidly fatal cases, and the urine of any patient, are not likely to be of value. Most of the other materials used have been collected at the time of the appearance of jaundice or only a day or two previously, that is, about five to ten days after the onset of symptoms in a disease with an incubation period of two to four weeks. Nasopharyngeal washings have not produced disease even in man, although they have been suspected to be a source of human infection. Duodenal juice may contain virus, but it is possible that if bile is present it may have a deleterious effect on the virus. (There is some doubt in my mind whether virus A grows in the liver.) Most of the specimens of blood which have been used have almost certainly contained some neutralizing antibody because they have been taken so long after the onset of infection. It is very likely that the most suitable materials for attempted transmission are blood collected no later than the second or third day of symptoms and stools collected at the same time or certainly no later than the first day of jaundice. The most suitable routines of administration for 10 per cent suspensions of stools without antibiotic or centrifugation are probably the oral route or injection directly into the liver and spleen. The intravenous and intraperitoneal routes are most suitable for blood or for highly concentrated sterile material prepared in the ultracentrifuge. (The sterile deposits from ultracentrifugation could also be used for the inoculation of eggs following prior injections of cortisone, but, of course, this work may already have been done.)

INFECTIOUS HEPATITIS

Canaries: The first suggestion that this species might be suitable for work in this field came from two different laboratories in Germany in 1943. Presumably, these birds were selected because they were readily available and cheap and not because of their color! Dresel and his colleagues in Dresden (Dresel *et al.*, 1943) reported that inoculation of urine, duodenal juice, or blood from hepatitis patients into the breast muscles of canaries resulted in sickness in one to two days and death in about four days. Pathological changes were observed in the parenchymal cells of the liver. There was no record of neutralization tests with acute and convalescent serum from the patients who provided the virus specimens, or from any other patients. Herzberg (1943, 1944) used a large number of canaries over a period of several years in similar investiga-

tions, and reported a probable transmission after an incubation period of 30 to 70 days. He also reported suggestive evidence of success in neutralization tests with convalescent serum from patients. However, Herzberg was rather cautious in his interpretation, and pointed out the susceptibility of these birds to infections of all kinds and the possibility of activating their own latent protozoal and virus infections during passage of tissues by direct inoculation. Wildführ (1953) has also reported extensive experiments with canaries in Leipzig, but his results are no more satisfactory than those previously reported. My own experience with a relatively small number of these birds was that they reacted poorly to the inoculation of sterile human urine or blood. Certainly these birds do not merit further attention.

Domestic Fowl, Including Pigeons: So many of these birds may be carrying viruses of the ornithosis group that they are unsuitable for experiment unless a known virus-free stock is used and the birds are rigorously isolated after birth. The few recorded attempts at transmission in these birds have been unsuccessful except for the brief report from Lucké of the production of an illness in ducklings, which apparently never received confirmation.

Guinea Pigs: A number of workers have reported questionable results suggesting that some abnormal condition had been produced in a very small proportion of the inoculated animals, but that no recognizable disease could be propagated in series under the conditions of the experiments (Findlay *et al.*, 1931; Cameron, 1943; Jersild and Krag, 1948; Melnick and Ward, 1948; MacCallum *et al.*, 1951). In addition, Verlinde and Boer in Holland (1943) reported the occurrence of fever and fatty degeneration of the liver in guinea pigs inoculated with blood or urine, and the disease was considered to be transmissible in series by inoculation of the blood or liver of the guinea pigs. Subsequent attempts to repeat their first work with EK-Seitz filtrates of feces and duodenal juice were relatively unsuccessful (Verlinde and Boer, 1948). The Dutch workers pointed out that the guinea pigs in their earlier experiments were on a very meagre diet, and suggested that this may have made them more susceptible than those used in the later experiments. Many other workers have obtained completely negative results in these animals. It is impossible to decide whether Verlinde and Boer were successful in their early experiments, which seems doubtful, or whether they were passing some latent guinea pig virus. Certainly, the large volume of negative results suggests that latent viruses which might give rise to confusion are uncommon in guinea pig stocks, and the further use of these animals under varying conditions, such as intravenous inoculation of pregnant females, might be of value.

Rabbits: Apparently these animals have been completely refractory to infection with human hepatitis, but the fact that the virus may have been propagated in cultures of the livers of newborn rabbits (Henle *et al.*, 1950) suggests that further investigations might be performed in

rabbits in utero or in newborn rabbits, if this has not already been done.

Dogs: These animals have been used without success and, in view of the widespread presence of canine hepatitis virus which may exist in a latent form, dogs are considered unsuitable for investigation of human hepatitis viruses.

Cats: Transmission of infectious hepatitis to cats has been unsuccessful in at least four different laboratories, and, because of the latent viruses which they carry and their susceptibility to viral and bacterial infections of other kinds, they are not very suitable for further investigations of hepatitis.

Swine: This species, whose gastrointestinal tract is similar to that of man, received a great deal of attention as a result of the original report by Andersen from Denmark in 1937, but it now seems extremely doubtful whether Andersen did, in fact, produce any lesions in the livers of his pigs, which were on a protein-deficient diet. Many other workers have recorded negative results in these animals, but it is not possible to determine from the literature whether adequately large series of animals have received known infected material by oral or parenteral routes. However, swine are unsatisfactory animals to handle in the ordinary laboratory, and it is doubtful whether they merit further attention.

Ferrets: These animals have received relatively little attention as far as their susceptibility to human hepatitis viruses is concerned. Although only negative results have been recorded, it is questionable whether they have been adequately explored. In our investigations in 1943 and 1944, there was great difficulty in obtaining animals free of tuberculosis and other infections, and we had to abandon their use. Clean stocks have now become expensive, but I think that they should be tried on a large scale by at least one laboratory.

Horses and Sheep: Both these species have been used in relatively small numbers, and there has been no suggestion that they are susceptible. There is no apparent reason why they should be used on a larger scale.

Thus, in summary, unless tissue cultures or chick embryos yield a satisfactory answer soon there is probably some justification in making additional attempts to produce disease in guinea pigs, rabbits, and ferrets by feeding and by parenteral injection of concentrated preparations of carefully selected materials from patients with infectious hepatitis.

RECENT EXPERIMENTAL WORK IN EUROPE

Circular letters were sent to several laboratories which were known to have worked on this problem in the past. In Leiden, Professor Verlinde confirmed that no more positive results had been obtained in guinea pigs in his laboratory. Dr. Wilterdink (1953), in collaboration with Professor Verlinde, has carried out further attempts to infect rhesus and cynomolgus monkeys with various materials, mainly stools

from children in the acute stage of the disease. The inoculum was treated with antibiotics until bacteriologically sterile, and injected intramuscularly in 5 to 10 ml. amounts. Cortisone, ACTH, and 2,2-bisparachlorophenyl-1,1-dichlorethane (which produced necrosis of the adrenal cortex in dogs) were injected before and at the time of the fecal inoculation in attempts to increase the susceptibility of the animals. Groups of only two to four monkeys were used, and no abnormality was seen on histological examination of tissues obtained when monkeys were killed 14 to 48 days after inoculation.

Dr. Lépine, in Paris, provided the following information concerning studies in his labortory:

The infecting materials he used, either stools or duodenal juice, were obtained from patients in the first day of recognized jaundice or were sera, the inoculation of which had been followed by jaundice. It was not possible to perform any experiments in man. Attempts to transmit infection were made in rhesus, cynomolgus, and cynocephalus monkeys, mice, ferrets, and fertile hens' eggs. The monkeys were inoculated intraperitoneally and intracerebrally, the mice intraperitoneally, intracerebrally, and intrahepatically, and the ferrets, intraperitoneally, intracerebrally, and intranasally. The same inocula were used in all of these animal species, but all experiments, including passages with tissues from a few animals that died, gave negative results. The same infecting material, plus antibiotics, was inoculated onto the chorio-allantoic membrane, and into the amnions and yolk sacs of embryos of different ages. There were a number of nonspecific deaths from some inocula, but this finding could not be reproduced on passage of various egg materials. Dr. Lépine observed that these specimens appeared to be more toxic for chick embryos than did stools from patients with poliomyelitis. Three puppies were also inoculated intraperitoneally with pooled feces, but all remained well.

Dr. Wildführ of the Karl Marx Institute in Leipzig sent reprints of his work on presumed transmission of the disease to hamsters, canaries, and chick embryos, but the results were not very convincing, and the author himself concluded that he had not found a suitable experimental host.

Dr. Essen, now at Eutin in Schleswig-Holstein, in collaboration with Professor Lembke, is still convinced that he is propagating the virus in chick embryo by inoculation onto the chorio-allantoic membrane. He believes that he can see the virus, which has a size of 180 $m\mu$, as measured in electron micrographs, both in stool filtrates and egg material. Most of the inoculated embryos die, and contain the virus particles, but virus can also be seen in the allantoic fluid and the chorio-allantoic membrane of the surviving embryos. Dr. Essen claims to have seen the virus in a filtrate of stool obtained from a patient 17 months after the onset of the illness. He considers that he has infected humans with his egg material. The same particles already mentioned have been seen in stools from patients with serum hepatitis and in the egg cultures

of the blood of these patients. Therefore, he believes that infectious hepatitis and serum hepatitis are the same disease. Dr. Essen considers that the reports of Henle and his colleagues merely confirm his own work, which he first published in 1944. Examination of Essen's material in some other laboratories should help to confirm his ideas or other workers' doubt of his claims of successful transmission.

In England, practically the only work done in recent years has been carried out by Andrewes, Dick, Gledhill, and Niven, at the National Institute for Medical Research. They have failed to produce a disease related to the inoculum from man in various experiments in mice and rats, but have thoroughly studied a disease in mice which they have termed mouse virus hepatitis (MVH), which has been reported separately in this Symposium.

Dr. Gunnar Olin, of Stockholm, reports that several years ago he tried to obtain positive complement-fixation using extract from the livers of patients with acute hepatitis who had died during the first month of hepatitis. The livers from ten patients, some of whom had died only a week to ten days after the onset of the disease, were tested, but all results were negative.

More recently, Dr. Knut Alin, in Dr. Olin's laboratory, has tried to repeat Henle's experiments with a skin-test antigen. Instead of inactivating the material with ultraviolet irradiation, he used 0.1 per cent formol at 4° C. for ten days. Only a few skin reactions were elicited, and these were not limited to patients with hepatitis.

Dr. Alin has also tried to cultivate the virus in roller-tube cultures on human embryonic tissue in bovine and amniotic fluid. The cultures were inoculated with serum or feces from patients in the acute phase of hepatitis. Some of the cultures were run for three months, the fluid being exchanged once or twice weekly. No cell degeneration occurred.

The fluid from some of the cultures run for two or three months was concentrated 50 to 80 times by ultrafiltration. Such concentrates are being used as antigen in complement-fixation tests against hepatitis sera. Partial fixation was obtained in sera in dilutions of 1:10 to 1:20, from some patients in the convalescent phase. Sera from the acute phase or taken some time after the disease showed less fixation or none at all. The number of sera tested has been small, but the results have encouraged further work.

MacCallum has carried out a small number of experiments on three different lines: (1) Attempts were made to demonstrate an interference phenomenon in mice and rats between materials suspected or known to contain hepatitis virus A and Rift Valley Fever virus. It is realized that the lesions caused by the two viruses in man are quite different, but if both attack the reticuloendothelial system, some interference might be demonstrated. (2) Because of the suggestion that elderly females have had a more severe hepatitis than others in certain outbreaks of the human disease, it was decided to use female rats which had had

four litters and were nine months to a year or more old. A few elderly males were also used. These rats were given 5 to 10 mg. of cortisone 24 hours before and immediately before the oral administration of a 10 per cent suspension in saline of untreated human stools from two patients in the pre-icteric stage of the disease. An elevated temperature response was obtained in a number of the rats after 12 to 16 days. Animals were killed at varying intervals. No abnormality of the organs was seen in the gross examination, and microscopic examination has not yet been done. The large intestine and caecum with their contents have been passed by mouth without antibiotics, with negative results. The other tissues remain frozen awaiting passage. A second experiment with stools from two other patients did not produce any temperature rises, and animals killed one to six months later showed no macroscopic abnormality. Further work along these lines is in progress. (3) About one month ago investigations of the possible interfering effect of hepatitis viruses on ascites tumors were begun. A small number of preliminary experiments in mice with serum or stool concentrates prepared in the ultracentrifuge have not indicated any inhibitory action on tumor growth.

A quite different type of investigation was carried out by Bauer in London about 1944, and seems worthy of mention in case others are commencing work in this field. (1) Rabbits were given daily subcutaneous injections of icterogenic serum until the Arthus phenomenon appeared. Injections of duodenal juice from cases of infectious hepatitis were then given, with the object of detecting a common antigen. The results were negative. (2) Immature female guinea pigs were sensitized to icterogenic serum (virus B). Some weeks later the uteri were set up in an isolated organ bath and normal serum was added. An anaphylactic response was obtained. Icterogenic serum or duodenal juice was then added with the object of detecting an antigenic substance not present in normal serum. In four experiments an anaphylactic response was obtained. In six experiments the preparation was unsatisfactory, and in two more, no response was obtained. This work was carried out at a time when Bauer considered the probability that the two agents were the same, but further reports suggesting that two different agents were concerned caused him to terminate these experiments. Bauer also carried out experiments in mice with the same unsatisfactory results which many others have had. After subcutaneous injection of serum, liver and spleen passages were carried out with further injection of serum. Lesions were caused in the liver, but the same results were obtained by the injection of normal mouse and human serum and extracts of mouse and human liver. The work was then stopped, but it seems likely that the stock of mice in use was carrying a latent hepatitis virus.

SERUM HEPATITIS

The position here is somewhat different from that in infectious hepatitis because the only known source of infectious material, if we

consider this disease to be the result of the action of a virus, is blood or its derivatives. There now seems to be considerable doubt whether antibodies to this agent appear in the serum in the same manner as they do in infectious hepatitis. Thus, known icterogenic pools which have given relatively high attack rates in man should be suitable for experimental work in animals. There are few, if any, recorded attempts to use material which has been concentrated in the ultracentrifuge, although I realize that this has probably been done. McCollum's experiments in filtration, which suggest a size of less than 50 mμ for the serum hepatitis agent, indicate that centrifugation should provide good starting material. The agent has proved so stable that it could probably be washed with saline or distilled water a sufficient number of times to remove any possible antibody without causing any appreciable loss of virus activity.

Most of the same species of animals have been used as for infectious hepatitis and, of course, all the results have been negative. As the mechanism of action of the agent concerned in man is not known, it is difficult to determine whether more work in animals is justified; very large groups of animals would be needed to start the experiment in order to make observations over a length of time comparable to the long incubation period of the natural disease in man. Because of the occurrence of homologous serum jaundice in horses, this species appeared to be worthy of investigation, but such experiments as have been carried out have not suggested that horses are susceptible to the agent causing serum hepatitis in man. However, as the mechanism of the horse disease is not known either, further investigation of this phenomenon might be of value.

References

1. Colbert, J. W., Jr.: Review of animal experimentation in infectious hepatitis and in serum hepatitis, Yale J. Biol. and Med. *21*:335-343, March 1949.
2. Dresel, E. G., Meding, G., and Weineck, E.: Über das virus der hepatitis epidemica, Ztschr. f. Immunitätsforsch. u. exper. Therap. *103*:129, 10 June 1943.
3. Essen, K. W., and Lembke, A.: Über der erreger der hepatitis epidemica, Zentralbl. Bakt. *159*:387-394, June 1953.
4. Herzberg, K.: Der kanarienvögeln als versuchstier in der hepatitis contagiosaforschung, Klin. Wchnschr. *22*:676-677, 30 Oct. 1943.
5. Herzberg, K.: Über tragungsversuche au kanarienvögeln mit hepatitis-contagiosa-material, Zentralbl. Bakt. *151*:81, 28 Jan. 1944.
6. Hodgman, S. F. J., and Laris, N. M.: Vet. Red. *65*:447, 1953.
7. Jordan, R. T., Nungester, W. J., and Preston, W. S.: Recovery of the Novy rat virus, J. Inf. Dis. *93*:2, 124-129, Sept.-Oct. 1953.
8. Novy, F. G., Perkins, W. A., Chambers, R., and DeKruif, P. H.: The rat virus, J. Inf. Dis. *93*:2, 111, Sept.-Oct. 1953.
9. Verlinde, J. D., and Boer, H. D.: Nederl. tijdschr. geneesk. *87*:1304, 1943.
10. Verlinde, J. D., and Boer, H. D.: Arch. ges. Virusforsch. *4*:1, 1948.
11. Wildführ, G.: Ztschr. ges. inn. Med. *8*:1, 1953.

Dr. Paul then noted that since 1947, energetic attempts have been made in Dr. Smadel's laboratory to transmit human hepatitis to a wide variety of animal species, and called on Dr. Morris to discuss this work.

REPORT ON A SERIES OF ATTEMPTS TO ADAPT THE VIRUSES OF HUMAN HEPATITIS TO A VARIETY OF EXPERIMENTAL ANIMALS

DRS. J. ANTHONY MORRIS, JOHN R. O'CONNOR, AND DON R. COBURN

The history of attempts by various workers to propagate the viruses of hepatitis of man in hosts other than human beings has been reviewed by others during this conference. The present study represents an investigation of different animal species, including selected species of North American wildlife, for their susceptibility to infection by the two viruses causing human hepatitis. Although these attempts have been unsuccessful, in keeping with this Symposium, the observations are documented for the record.

MATERIALS AND METHODS

Human Hepatitis Virus A. Serum samples from 57 clinical cases of infectious hepatitis occurring among American soldiers stationed in the Far East were obtained on the fourth to 49th day after onset of illness. The samples were stored under solid CO_2 refrigeration for six to 24 months prior to use in these experiments. The pooled Seitz filtrates of stools obtained from three cases of infectious hepatitis were provided by Dr. Balduin Lucké for use in some of the tests in pigeons. The presence of hepatitis virus A in the plasma and the stools was not ascertained, since none of the specimens was inoculated into volunteers.

Human Hepatitis Virus B. Five batches of human plasma or serum were used as the seed materials; four of these were known to be infected with human hepatitis virus B. Batch 034 was obtained through the kindness of Dr. W. H. Bradley, Ministry of Health, London (Bradley, 1946). This lot of dried serum was prepared in 1944 and was shown to be icterogenic two years later by inoculation into human volunteers. It was received in our laboratory in 1947, when it was rehydrated and stored at —70° C. until used in the present experiments during the ensuing five years. Lot 310 was kindly furnished by Dr. Eugene R. Sullivan, Division of Biologic Laboratories, Massachusetts Department of Health. This material, a portion of which had been administered to seven patients of whom five developed typical hepatitis (Sullivan, personal communication), was received in our laboratory in the frozen state in 1947. It was kept at dry ice temperature for periods ranging from one month to five years before its use in these studies. Plasma pool NIH-8 and two other plasma pools were obtained from Dr. R. Murray, Laboratory of Biologics Control, National Institutes of Health, Bethesda, Maryland. These were maintained in storage in Dr. Murray's laboratory for several years at —17° to —20° C., and in our laboratory at —70° for several months until used. Pool NIH-8, the detailed history of which has been reported (Murray, *et al.*, 1953), is known to be in-

fectious in human beings in a dilution of 10^{-4}. Dr. Murray kindly supplied the following data pertinent to the remaining two pools: Plasma from three patients in the pre-icteric phase of homologous serum jaundice made up one pool. These specimens were obtained 22 days following inoculation of infected plasma into three volunteers. The plasma specimens making up the other pool were collected from the same three volunteers 54, 59, and 90 days following inoculation, when each donor was icteric. The 90-day specimen was shown to be infectious by inoculation into 15 human volunteers, three of whom developed hepatitis with jaundice. An additional inoculum, provided by Balduin Lucké and destined for some of the pigeons, consisted of pooled Seitz filtrates of liver from two fatal cases of serum hepatitis.

Animals Employed. Three strains of inbred mice, laboratory-raised guinea pigs and white rats, and pen-raised and trapped wild animals were employed. The inbred mouse strains were the C57 (black), a black and tan mutant derived from C57 (black), and A albino, subline L. The laboratory-bred animals were generally young adults, unless otherwise specified, when inoculated. North American wild animals were selected for use on the basis of availability and the ease with which they could be handled and bred. The mastomys used in these tests were obtained from a colony of these rat-like rodents established at the Army Medical Service Graduate School from parent stock supplied by Dr. D. H. S. Davis, Plague Research Laboratory, Department of Health, Johannesburg, South Africa.

Test procedure A. Initial efforts were directed to attempt the adaptation of human hepatitis B virus by serial passage so that it would produce obvious disease in an experimental host. The seed material was inoculated into six animals of a species by the following routes: intracerebral, intraperitoneal, intranasal, and subcutaneous. From these animals three series of blind passages were established at intervals of 14, 30, and 60 days, respectively, following the date of initial inoculation. In a few instances the intervals between inoculation and tissue harvest were five, ten, and 20 days. The passage material consisted of bacteriologically sterile 10 per cent combined organ suspensions (brain, liver, kidney, heart and spleen) in 10 per cent normal rabbit serum. This was inoculated into six animals of the same species by the same routes used for the initial inoculum. Two of the six animals which had been inoculated with material from the first passage animals were sacrificed at the end of the appropriate period for further passage, and the remainder was kept under observation for periods up to 60 days. Two or more passages were made in each series before a species was classed as refractory. All sacrificed animals were dissected and their tissues examined grossly. If lesions were observed, histological sections were made.

Test procedure B. Fifty-seven human serum specimens obtained four to 49 days after onset of clinically diagnosed infectious hepatitis were tested in the following manner for presence of an agent pathogenic

in a few selected avian and rodent species: Four to six individuals of a species were inoculated by multiple routes with a serum sample, and the inoculated animals observed for periods up to 60 days. Passages were made only if obvious illness developed in the inoculated animals.

RESULTS

The animals which were tested by procedure A for susceptibility to infection by human hepatitis virus B are listed in Table I.

TABLE I

ANIMALS FOUND REFRACTORY TO INFECTION BY HUMAN HEPATITIS VIRUS B

MAMMALS	
Cat (Felix domestica)	Opossum (Didelphis virginiana), suckling
Chipmunk (Tamias striatus)	Opossum (Didelphis virginiana), adult
Dog (Canis familiaris)	Prairie dog (Cynomys sp.)
Ferret (Mustela furo)	Raccoon (Procyon lotor)
Fox (Vulpes fulva)	Rat, albino
Guinea pig	Rat, cotton (Sigmodon hispidus)
Swine	Rat, kangaroo (Dipodomys sp.)
Mink (Mustela vison)	Rat, pack (Neotoma sp.)
Mouse, Swiss, suckling	Rat, rice (Oryzomys palustris)
Mouse, Swiss, 8-10 gm.	Squirrel, gray (Sciurus carolinensis)
Mouse, A albino, subline L	Squirrel, ground (Citellus sp.)
Mouse, gray (Mus musculus)	Vole (Microtus sp.)
Mouse, white-footed (Peromyscus leucopus)	Woodchuck (Marmota monax)
BIRDS	
Canary (Serinus sp.)	Pheasant (Phasianus colchicus)
Duckling (Anas moschata)	Quail (Colinus virginianus)
Pigeon (Columba sp.)	Starling (Sturnus vulgaris)

It was not possible to produce obvious hepatitis or any transmissible disease attributable to human hepatitis virus B in any of these species. The only suspicious lesions observed were encountered in pigeons (inflammatory and degenerative changes in the liver) and in Swiss mice (focal mononuclear infiltrations with necrosis of the parenchymatous cells of the liver, associated with moderate to severe ascites). The lesions observed in inoculated pigeons were shown to be the result of infection with either psittacosis virus or Salmonella typhimurium.

The presence of psittacosis infection was confirmed by isolation of this virus in mice and eggs inoculated with tissues from the test pigeons, or by demonstrating the presence of complement-fixing antibodies against psittacosis in the serum. The distribution of psittacosis infection in a typical blind-passage experiment in pigeons is shown in figure 1. Tissues from other pigeons yielded Salmonella typhimurium when cultued on blood agar; these were identified with type-specific sera.

Additional attempts were made to transmit the hepatitis virus to pigeons apparently free of psittacosis and Salmonella. These were ob-

tained as follows: Five male and 15 female pigeons were separated from a flock known to be infected with psittacosis and Salmonella. Each of these isolated pigeons was given 125 milligrams of chloromycetin by mouth and 150,000 units of penicillin G in oil intramuscularly daily for five days. Two months after treatment the sera from three of the pigeons gave positive complement-fixation tests for psittacosis; these birds were discarded, and the antibiotic treatment was repeated in the remainder of the flock. Ten months after the second treatment all breeders and squabs in the flock were negative for psittacosis complement-fixing antibody. Neither psittacosis nor Salmonella was recovered from the tissues of 19 pigeons from this flock which have been used in passage experiments.

Six of these pigeons were inoculated intravenously with 1.0 cc. of pooled Seitz filtrates of 20 per cent liver suspensions from two fatal cases of serum hepatitis. Four other pigeons were similarly inoculated with Seitz filtrates of stools from three clinical cases of infectious hepatitis. Two pigeons of the group of six were sacrificed on the 14th, 30th and 60th day after inoculation; two pigeons of the group of four were sacrificed on each the 14th and 30th day. None of these pigeons exhibited lesions suggestive of hepatitis. No serial passages were undertaken with materials from birds of these two groups.

Our negative findings in pigeons are unlike the results of Lucké and Ratcliffe, (1949) who in various experiments injected pigeons intravenously with blood plasma or Seitz filtrates of feces of active cases of hepatitis and two to three weeks later found evidence of liver disease characterized by extensive necrosis of liver cells and inflammatory reaction around the portal canals.

Ascites and hepatosplenomegaly were observed at the Army Medical Service Graduate School in Swiss mice originally inoculated with hepatitis virus B materials, and later in the same strain of mice inoculated with suspensions of "normal" mouse tissues or with materials from hemorrhagic fever patients (Morris, 1954). This disease has been found to be associated with cytoplasmic structures resembling protozoa. The disease in our mice was not observed by these authors.

Procedure B, as outlined above, was employed in tests with hepatitis virus A specimens in the form of serum obtained four to 49 days after onset of illness from 57 patients with clinically diagnosed infectious hepatitis. These specimens were tested for an agent that might be pathogenic for one or more of eight selected species of animals. It was hoped that such a wide selection of presumably infectious material might provide an agent with unusual pathogenicity for one of the host species, and that this variant might induce overt disease in one of the inoculated experimental hosts. The number of serum specimens tested in each of the animal species is shown in table II. None of the serum samples employed resulted in the establishment of a transmissible disease agent in any of the inoculated animals.

TABLE II

SERA OF CASES OF HUMAN INFECTIOUS HEPATITIS TESTED IN INDICATED HOSTS

Day of Disease Serum Taken	Total Specimens Tested	Number of Serum Specimens Tested in									
		Birds		Inbred Mice				Other Rodents			
		Quail	Pheasant	A/L	C_{57}	B & T	Deer-mouse	Microtus	Hamster	Mastomys	Guinea Pig
4-6	11	7	0	10	4	0	4	2	9	6	3
7-49	39	32	11	34	5	1	10	20	14	27	11
Unknown	7	6	2	6	5	3	0	1	5	6	1

DISCUSSION

The results of the present studies differ from the published reports of successful propagation of the viruses of human hepatitis in canaries (Herzberg, 1943), swine (Andersen and Tulinius, 1938), guinea pigs (Verlinde, 1946), and pigeons and ducks (Lucké and Ratcliffe, 1949).

The majority of the inocula used in the current hepatitis virus B studies were shown to have contained virus prior to our experiments, since humans inoculated with four of the five plasma pools manifested symptoms of hepatitis. It appears significant, therefore, that in none of these pools was an agent demonstrated that was pathogenic for the several species of animals employed. Our negative results with autopsy material from fatal cases of serum hepatitis and with serum and autopsy tissue from cases of infectious hepatitis are less significant than those with the hepatitis B sera, since in none of the former materials was virus shown to be present by demonstrating infectivity for man.

SUMMARY

Twenty-three mammalian and six avian species were tested in blind-passage experiments for susceptibility to the agent of human hepatitis virus B. It has not been possible to establish an obvious disease attributable to this virus in any of these species.

Pigeons apparently free of Salmonella and psittacosis infection did not develop significant lesions following intravenous inoculation of either suspensions of stools of patients in the acute phase of hepatitis virus A infection or suspensions of liver from fatal cases of hepatitis virus B infection.

A transmissible disease characterized by hepatosplenomegaly and ascites and associated with intracellular bodies resembling protozoa was encountered in Swiss mice originally inoculated with human material as well as in control mice of the same strain inoculated with suspensions of normal mouse tissue.

None of 57 serum specimens obtained from patients at varying times after onset of clinically diagnosed infectious hepatitis contained an agent pathogenic for any of several species of animals.

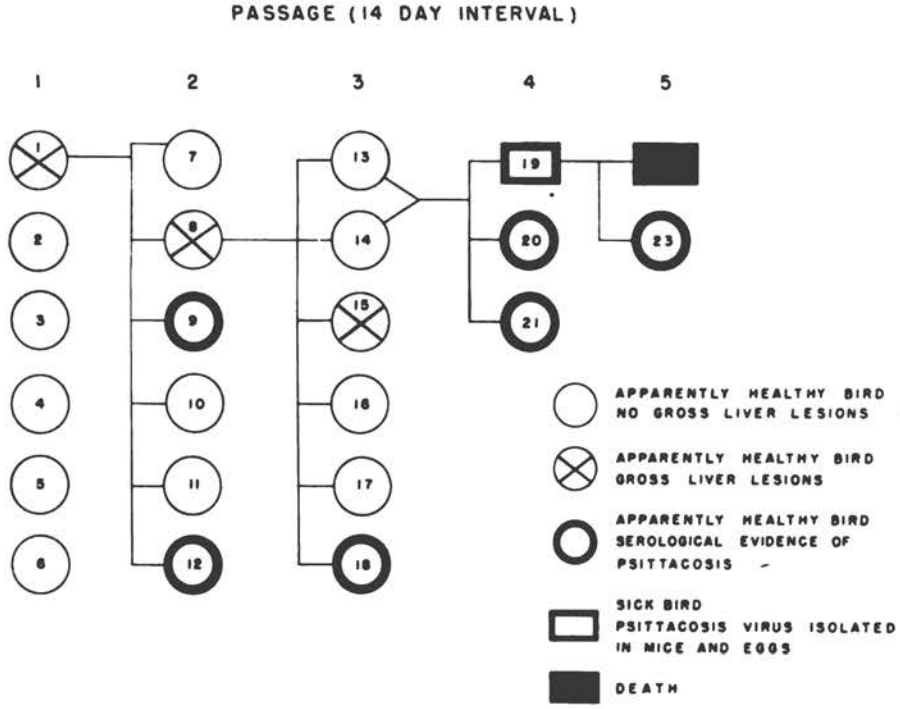


FIG. 1. Distribution of psittacosis infection during blind passage of hepatitis virus in pigeons.

References

1. Andersen, T. T., and Tulinius, S.: The etiology of hepatitis epidemica, Acta. med. Scandinav. *95*:497-509, 1938.
2. Bradley, W. L.: Homologous serum jaundice, Proc. Roy. Soc. Med. *39*:649-654, Aug. 1946.
3. Herzberg, K.: Der kanarienvögeln als versuchstier in der hepatitis contagiosa-forschung, Klin. Wchnschr. *22*:676-677, 30 Oct. 1943.
4. Jordan, J., and Mirick, G. S.: Hepatitis in mice of presumed viral origin, Bull. Johns Hopkins Hosp. *89*:328-331, Oct. 1951.
5. Lackey, M. D., Eichman, P. L., and Havens, W. P., Jr.: Hepatitis in laboratory mice, J. Inf. Dis. *93*:1, 14-20, July-Aug. 1953.
6. Lucké, B., and Ratcliffe, H.: Virus hepatitis of birds and its possible relation to epidemic hepatitis of man, Tr. A. Am. Physicians, *62*:83-88 1949.
7. Morris, J. A.: Ascites and hepatosplenomegaly in mice associated with protozoan-like cytoplasmic structures, Bact. Proc. 1954.
8. Murray, R., Diefenbach, W. C. L., Ratner, F., and Leone, N. C.: Experimental investigation of homologous serum hepatitis, Am. J. of Publ. Hlth. *42*:9, Sept. 1952.
9. Sullivan, E. R.: Personal communication.
10. Verlinde, J. D.: Experimentelle hepatitis epidemica, Nederl. tijdschr. v. geneesk. *90*:1309-1312, 5 Oct. 1946.

DISCUSSION

Dr. Paul thanked Drs. MacCallum and Morris for their presentations, and then asked whether further studies had been made using the material with which Dr. Lucké had reported initial success in propagating human hepatitis in ducks and pigeons.

Dr. Morris reported that in 1952 he had received from Dr. Lucké samples of the materials obtained after passages through ducks and through pigeons. These materials were given intravenously to newly hatched ducks and pigeons, but liver lesions did not develop. Although the weight patterns of the inoculated birds were not followed as Dr. Lucké had followed them, the studies were considered unsuccessful when liver lesions failed to appear.

Dr. Werner Henle mentioned that he had unsuccessfully attempted to infect young ducklings with the IH and SH materials available in his laboratory, some of which were the materials originally used by Dr. Lucké. He added that Dr. Lucké's studies had not included neutralization tests with human convalescent sera.

Dr. Stokes noted that since definite neutralization tests had not been included in Dr. Lucké's study, it had not seemed justified to use the duck and pigeon material in human transmission experiments.

Dr. Murray said that he could add to Dr. MacCallum's excellent review of current European work the information that reports in the Russian medical literature reflect considerable interest in propagating the agent of human hepatitis which was referred to as "Botkin's Disease." V. Schwartz had claimed recently* that the disease had been passed to guinea pigs by administration via stomach tube of duodenal fluid obtained from human cases in the acute phase. However, the description of the illness produced in the experimental animals was not sufficiently clear, and the claims were of questionable validity.

Dr. Enders expressed interest in the attempts described by Dr. MacCallum to produce anaphylaxis with hepatitis virus. In his own laboratory, Dr. Enders had unsuccessfully tried to produce a generalized anaphylactic response by giving mumps virus intravenously to guinea pigs which had previously received mumps antiserum. The idea of sensitivity to a virus was a provocative one, but he wondered whether there had been experiments in which viruses other than the tobacco mosaic virus had been proved to act as anaphylactogens.

Dr. Sabin replied that in 1931 he had attempted to sensitize the guinea pig uterus with poliomyelitis virus. The virus was concentrated to a titer of about 10^{-3} or 10^{-4} and purified by adsorption and elution. The purpose of the study was to determine the value of the anaphylaxis test in detecting minimal residual amounts of monkey spinal cord protein in the virus preparation. However, the uteri of guinea pigs which had received previous injections of the virus did not react.

---

* Klin. Med. Moskva, *29*:51-54, 1951.

Dr. MacCallum then stated that it was not readily apparent from published reports just how far serum hepatitis had been carried by serial passage in man, and asked if this point could be clarified.

Dr. Werner Henle commented that the dilute whole blood mentioned previously, of which 1/20,000 cc. was calculated to be infectious, had been obtained from volunteers inoculated with second-passage Fort Bragg serum. The serum from the patients infected with this whole blood was again used for preparing a pool which was subsequently proved to be infectious. This sequence, therefore, represented one instance of four serial passages of SH.

Dr. Mirick added that at the Great Lakes Naval Training Station after the war, patients with streptococcal infections were being treated with pooled serum from individuals convalescent from streptococcal infections. It became apparent, after five consecutive passes of such pooled convalescent serum, that the recipients were becoming ill with hepatitis. This experience, therefore, represented five inadvertent serial passages of the icterogenic agent.

Dr. Murray noted that Dr. Werner Henle had provided Dr. Oliphant with acute phase serum from patients inoculated with Fort Bragg virus for incorporation into the plasma pool designated as NIH-8. This pool produced hepatitis in volunteers, and acute phase serum from one of these volunteers produced hepatitis again when it was inoculated into a second group. All of these last cases were quite severe and had slightly shorter incubation periods.

Dr. Sabin asked if Dr. MacCallum still felt it possible, as he had hypothesized earlier, that the liver lesions of SH or IH are not caused by a virus, but depend on the presence of some adjuvant etiological agent stirred up by the presence of the virus in the gastrointestinal tract or the lymph nodes. Since the successful serial transmission studies had demonstrated that hepatitis can be produced by parenteral injection of the infected material alone, development of the disease did not seem to depend on simultaneous infection with bacteria or other possible adjuvants. The adjuvant agent for the production of liver disease would, therefore, have to be normally present in the body.

Dr. MacCallum replied that the theory had still not been disproved, and he had suggested the hypothesis to be provocative. The histological picture of hepatitis in the liver, with necrosis of parenchymal cells, closely resembles a toxic change, whereas other known virus diseases produce different and more specific hepatic pathology.

Dr. Dalldorf noted that histological changes comparable to those of infectious hepatitis also occur with yellow fever. He recalled that Dr. Tracy Mallory had once described findings in hepatitis livers which were difficult to distinguish from Councilman bodies.

Dr. MacCallum pointed out that different parts of the liver lobule are attacked by the two diseases. Hepatitis produces central necrosis, while yellow fever produces mid-zonal necrosis.

Dr. Sabin said that it would be difficult to distinguish pathologically between many "toxic" changes in the liver and the changes produced by viruses. In addition, with the frequent use of serial liver biopsies during the course of infectious hepatitis, pathologists had begun to correlate typical changes in the liver with specific phases of clinical hepatitis.

Dr. Paul recalled that the theory mentioned by Dr. MacCallum had certainly had some worthy advocates.

Dr. Sabin asked whether it was not true that Dr. Mallory had found typical abnormalities in specimens obtained by liver biopsy even in the early, pre-icteric phase of hepatitis.

Dr. Stokes replied that this was so. In addition, Dr. Mallory had noted changes in a high percentage of livers obtained from men who had suffered accidental deaths during a hepatitis epidemic, but who had had no symptoms of hepatitis before death.

Dr. Tillett noted that another peculiar aspect of serum hepatitis was the fact that the disease depended upon an artificial mode of transmission for its development. If there were so many carriers of the SH virus, it seemed strange that spontaneous cases did not occur. Perhaps spontaneous cases did actually occur, but were inapparent and unrecognized.

Dr. Havens agreed that serum hepatitis had been arbitrarily designated as a disease which occurred after artificial exposure. If a patient developed hepatitis with no history of antecedent inoculations, it was certainly difficult, despite a long incubation period and absence of contact with cases of IH, to say that his illness was not infectious hepatitis. This difficulty had arisen in two patients Dr. Havens had seen who had provided the single blood donations given to recipients in whom hepatitis developed three to four months after transfusion. The two donors themselves became ill with hepatitis eight months and one year, respectively, after making their last donations of blood which proved infectious. It seemed probable that these two men were SH carriers in whom the overt disease occurred spontaneously, although it was certainly conceivable that they had subsequently contracted IH.

Dr. Murray recalled that there had been several peculiar instances in which patients who were initially thought to have IH may well have had spontaneously occurring SH. Dr. Evans had reported a case which was thought to be IH, but when the patient's serum was inoculated into volunteers, it produced disease only after a long incubation period. In addition, Dr. Murray had seen a similar case of clinically classical IH, and this patient's serum, too, produced a disease characterized by a long incubation period. Dr. Murray agreed that it was extremely difficult in certain cases to determine whether the disease was IH or whether it was SH occurring spontaneously without artificial exposure.

Dr. Paul then called on Col. Gochenour to describe the virus diseases which cause hepatitis in certain species of animals.

III. REVIEW OF VIRUSES WHICH CAUSE HEPATITIS IN CERTAIN SPECIES OF ANIMALS

LT. COL. WILLIAM S. GOCHENOUR, VC USA

INTRODUCTION

This paper is a brief review of the known characteristics of certain viruses causing hepatitis of domestic animals. The purpose of this report is to discuss these agents in the light of their suitability as prototypes for the study of the viruses of infectious hepatitis and of serum hepatitis in man. Because of restrictions upon the importation of exotic pathogens, only viruses native to the United States will be considered. In addition to viral agents producing liver dysfunction and frank hepatic pathology, it appears desirable to include those animal viruses which possess characteristics common to this group.

The viruses to be discussed are the etiologic agents of equine influenza —virus abortion, equine infectious anemia, infectious canine hepatitis— epizoötic fox encephalitis, canine distemper, "hard pad" disease of dogs, feline panleucopenia, hog cholera, and raccoon jaundice.

These diseases can be roughly separated into two groups, the first possessing many of the characteristics of human infectious hepatitis, the second similar to serum hepatitis.

ANIMAL DISEASES RESEMBLING INFECTIOUS HEPATITIS

Equine Influenza—Virus Abortion: Equine influenza has long been recognized as a potentially explosive disease which, under the favorable conditions of high susceptible population density, causes great panzoötics in which nearly every equine in the area is infected. Until the advent of mechanized transportation the disease was a threat to the civilian economy and to military operations. Equine virus abortion was first recognized as a distinct disease entity by Dimock and Edwards in 1933. Despite intensive study of both diseases and the striking similarity of their morbid anatomy, it was not until the last few years that the identity of the two viruses was established.

When the central nervous system is not involved, both diseases are characteristically mild. The influenza form begins with a high fever of sudden onset which persists for several days, and as suddenly abates. Lacrimation, photophobia, and transient corneal opacity are almost constantly present. Jaundice is common, as are tendovaginitis, nephritis, and lymphadenopathy. Convalescence is usually complete by the end of three weeks. In the pregnant mare, abortion is commonly the only manifestation. Severe disease in both forms is manifested by weakness, muscle tremors, posterior paralysis, and prostration, and is commonly fatal.

The disease may be naturally acquired by ingestion of virus-contaminated discharges and fetal membranes or tissues, as well as by venereal

infection. Artificial infection is readily induced by parenteral injection of blood or tissue suspensions.

Following naturally acquired disease, animals develop an immunity of several years duration.

The virus is demonstrable in all fluids and tissues of acutely ill animals and of aborted fetuses. Virus has been demonstrated in the circulating blood months after clinical recovery, and in semen for reported periods of up to six years.

Inclusion bodies are found in the nuclei of parenchymal liver cells, in the epithelium of the pulmonary alveoli, in the bile ducts, in endothelial cells, and in the spleen, thymus, and lymph nodes.

The virus is relatively stable, surviving in blood for several months at refrigerator temperatures and for years at —70° C. Dessicated virus survives for at least ten months at temperatures up to 21° C.

The susceptibility of laboratory animals to this virus is not clearly established. Propagation of the virus other than in equines has been reported only in day-old hamsters, in guinea pig fetuses, and on human amnion grafted onto the amnion of the embryonated hen's egg.

Active immunity of several months' duration has been elicited by vaccination with formalinized fetal liver suspensions and with living spleen-virus suspensions.

Infectious Canine Hepatitis—Epizoötic Encephalitis: This disease of canines, also known as Rubarth's disease, was first recognized in foxes and its viral etiology established in 1930. Description of the artificially-induced disease in dogs was soon followed by recognition of the naturally occurring disease.

In the dog, the disease occurs in all age groups, but susceptibility and mortality is highest among puppies. Animals may die within hours after the onset of symptoms in the peracute form. Most commonly, death or recovery occurs within a fortnight. Clinically, the disease is manifested by an initial high fever, apathy, inappetence, vomiting, diarrhea, marked thirst, and abdominal pain. Tonsillitis is common. The oral mucosa is pale and often petechiated. Photophobia and lacrimation are common, and as in equine influenza, transient corneal opacity is not uncommon.

In foxes, the disease runs a short course, seldom lasting over 24 hours, and is generally fatal. Onset is sudden, characterized by violent convulsions and rapidly succeeded by apathy, paralysis, coma, and death. These manifestations are directly referable to hemorrhagic extravasations into the brain and cord, and not to viral attack of nerve cells.

The disease may be naturally acquired by contact with body discharges or urine contaminated with the virus, or artificially induced by parenteral inoculation of infective fluids or tissues.

Dogs recovering from naturally acquired infection develop a long-lasting and solid immunity. Such animals have been shown, however, to shed virulent virus in their urine for many months.

Inclusion bodies are acid-staining, and are demonstrable in the nuclei of endothelial cells in the liver, spleen, lymph nodes, and small vessels, as well as in the cord cells of the liver.

The virus is stable at refrigeration temperatures, and, like rabies virus, can be preserved at room temperature for protracted periods in neutral glycerol.

Ferrets, mink, and the common laboratory animals are not susceptible. Attempts to propagate the virus on embryonated eggs have not yet been successful. Successful cultivation has been reported in roller-tube culture of dog kidney tissue.

Passive immunization has been employed with success in puppies and foxes, using sera prepared in these animals. Active immunization with formolized virus-tissue suspensions has met with limited success. Dogs have developed immunity following simultaneous administration of virulent virus and antiserum. Such animals, however, became renal carriers and shedders of the virus. In foxes, this method resulted only in suppression of fatal infection until after the antibody received had been eliminated.

Canine Distemper: This virus disease is included in this discussion primarily as a contrast to infectious canine hepatitis. The disease is widespread among dogs throughout the world. Experience with the virus is so common that dogs susceptible to infection after one year of age are rare. Foxes, wolves, raccoons, and mink are natually infected. Ferrets are extremely susceptible and almost universally succumb. The virus has been successfully adapted to propagation in the embryonated egg.

Distemper virus is most commonly spread by droplet infection, although urine and discharges of infected animals are virus-contaminated.

Distemper viremia in dogs is of short duration, and has not been reported following convalescence. Inclusion bodies are demonstrable in the cytoplasm of the epithelial cells of the lung, gut, and bile duct, and in cells of the liver and of the reticuloendothelial system.

The virus is quite labile, being readily destroyed by heat or drying, though it may be successfully preserved by freezing and freeze-drying. Tissue suspensions have been inactivated by ultraviolet irradiation.

Immunization by the use of both killed spleen-virus suspensions and living modified viruses is successfully practiced, as is vaccination with a combination of killed and live virus.

Hard Pad Disease of Dogs: The subject of considerable disagreement among veterinary virologists, the agent of hard pad disease is generally accepted as being distinct from canine distemper virus. Other than the dog, only the ferret is known to be susceptible. The disease takes its name from a constant and characteristic hyperkeratosis of the foot pads. The significant changes, however, are those of a diffuse inflammatory demyelinating encephalitis. Histopathological changes are proliferation of glial cells and of the vascular endothelium.

The incubation period is approximately one week in dogs, and three weeks to one month in the ferret.

Intranuclear inclusion bodies are found in histiocytes, glial cells, and cells of the ependyma.

Dogs experiencing infection develop a high degree of immunity, though remaining susceptible to canine distemper. Dogs immune to canine distemper appear to react less severely to hard pad virus infection than do those not immune.

Feline Panleucopenia: This disease takes its name from the almost complete disappearance of circulatory leucocytes in early illness. Typically, the disease is an acute, febrile infection with a high mortality rate. Fever is usually diphasic. Vomiting, diarrhea, and rapid weight loss from dehydration are characteristic.

Felines are the only known susceptible animals. Artificial propagation has not yet been successful. The virus is relatively stable in glycerol, but is rather susceptible to drying.

Virus is demonstrable in the blood, feces, nasal secretions, and urine of infected cats. Intranuclear acid-staining inclusion bodies are found in epithelial cells of the gut, the parenchyma of the liver, and tubules of the kidney.

Transmission is usually by contact, but fleas have been shown to be significant vectors in catteries and laboratories.

Active immunization with formolized tissue virus is quite successful.

Hog Cholera: This extremely contagious, acute, and highly fatal disease of swine is primarily a disease of the small blood vessels, whose degeneration leads to hemorrhage, local necrosis, and infarction in the spleen and other organs. Neurotropism is evident in a large percentage of natural infections, and is manifested by perivascular "cuffing", glial proliferation, and degeneation of nerve cells, accompanied by round cell infiltration. Leucopenia is extreme in uncomplicated disease.

The virus reaches maximum concentration in the blood and tissues at the peak of the temperature rise, and is present in all discharges. Intranuclear inclusion bodies in the epithelial cells of the gall bladder have been reported.

Swine are the only known naturally infected species. The virus has been adapted to the rabbit, in which its only manifestation is elicitation of a febrile reaction. In addition, it has been propagated in swine testicular tissue cultures, on the chick chorio-allantois, in Tyrode's solution, and on serum agar slants.

The virus is estimated to be 35 $m\mu$ in size. It is quite susceptible to drying, but quite stable under refrigeration temperatures, or in glycerol or 0.5 per cent phenol. It is readily freeze-dried.

Significant transmission of infection is achieved by feeding raw pork scraps or by careless handling of blood or virus used for immunization.

Active immunity may be elicited by killed tissue vaccines, modified virus vaccines, or by simultaneous administration of antiserum and living virulent or modified virus.

Raccoon Jaundice: This recently described disease of raccoons appears to be due to a virus distinct from any of the forementioned animal viruses. The ferret is the only animal other than the raccoon known to be susceptible. Attempts to culture the virus on embryonated eggs have thus far been unsuccessful. Virus has been stored for six months at —70° C.

In summarizing this brief review of the animal diseases resembling infectious hepatitis, it would appear that the equine influenza—virus abortion, canine hepatitis,—epizoötic fox encephalitis, and raccoon jaundice viruses might provide suitable prototypes for study of human infectious hepatitis.

Unanswered questions at the moment concern host cross-susceptibility to the other viruses, virus behavior in the equine, and adaptation to other than the primary hosts. The fetal guinea pig passage technique and alternate passage in primary and secondary hosts seem worthy of further investigation. Cross-neutralization studies between these four viruses would seem desirable, as would further tissue culture study. Serological examination of paired human sera from known cases of infectious hepatitis for complement-fixing antibodies against these three viruses would appear a logical preliminary investigation.

ANIMAL DISEASES RESEMBLING SERUM HEPATITIS

This group is represented in this discussion by a single virus, that of equine infectious anemia. This disease was recognized as a clinical entity in the mid-nineteenth century, and its viral etiology was established in 1907. Despite world-wide study for almost half a century, information regarding the virus and its range of hosts is both inedaquate and conflicting. Consideration must be given the possibility that other infectious diseases of horses, notably equine leptospirosis, may have been responsible for this confusion. Nonetheless, certain reported characteristics of the virus and of the disease merit further investigation. This is the only virus of those discussed today which is reportedly pathogenic for man.

The disease in equines may be acute, subacute, or chronic. Apparent recovery from acute excerbations is followed by relapses at varying intervals, either spontaneous or provoked by fatigue. Viremia persists throughout the latent periods, which may extend over many years. Clinical manifestations are high fever, weakness, icterus, petechiation of the oral mucosa, rapid weight loss, and anemia. Pathological findings include hemorrhages in the parenchymatous organs, splenomegaly, hemosiderosis of the liver sinusoids, and replacement of yellow marrow by red marrow in the long bones.

European reports of experimental infection in swine describe an acute febrile disease, occasionally fatal, with a viremia persisting months after recovery.

Infection of the rabbit is said to result in an initial leucopenia and anemia, followed at two to four weeks by a transient fever and some loss of weight.

A reported accidental human infection resulted in a disease manifesting fever, headache, fatigability, weight loss, anemia, and diarrhea. Relapses occurred with increasing frequency. Viremia was demonstrated by animal inoculation to persist for at least three years. The similarity of these observations to severe human serum hepatitis is highly suggestive.

The virus is estimated to be between 18 and 50 mμ in diameter. It is inactivated by heating at 60° C. for one hour. It is destroyed by sunlight, but is quite resistant to chemicals, putrefaction, and drying.

Natural transmission of the disease is mediated by fly and mosquito vectors, as well as by contaminated syringes and tattooing equipment. The infectivity of the virus by oral routes is questionable.

In view of the above, it would appear desirable to attempt to establish the relationship, if any, of the virus of serum hepatitis to that of equine infectious anemia. In the literature available to the author, inoculation with serum hepatitis virus of equines proven to be susceptible to infectious anemia has not been reported. This would appear to be a simple and direct method of determining their possible relationship, and might well determine the suitability of this disease as a prototype for the study of serum hepatitis in man.

References

1. Cabasso, V. J., Stebbins, M. R., Norton, T. W., and Cox, H. R.: Propagation of infectious canine hepatitis virus in tissue culture, Proc. Soc. Exper. Biol. & Med., *85*:239-245, Feb. 1954.
2. Hagan, W. A., and Bruner, D. W.: The infectious diseases of domestic animals, 2nd edition, Comstock Publishing Co., Ithaca, N. Y., 1951, pp. 761-801, 823-825, 830-839.
3. Hoskins, H. P., LaCroix, J. V., and Mayer, K.: Canine medicine, American Veterinary Publications, Evanston, Ill., 1953, pp. 480-498.
4. Hutyra, F., Marek, J., and Manninger, R.: Special pathology and therapeutics of the diseases of domestic animals, Alexander Eger, Inc., Chicago, Ill., 5th English edition, 1946, Vol. III, pp. 74-100.
5. Kilham, L., and Herman, C. M.: Isolation of an agent causing bilirubinemia and jaundice in raccoons, Proc. Soc. Exper. Biol. & Med. *85*:2, 272-274, 1954.
6. Rivers. T. M.: Viral and rickettsial infections of man, J. B. Lippincott Co., Philadelphia, 2nd edition, 1952, 668-669.

DISCUSSION

Dr. Paul thanked Col. Gochenour for his review, and then called on Dr. MacCallum for further discussion of animal viruses which cause hepatitis.

Dr. MacCallum pointed out that Miles *et al.* had recently reported[1] successful propagation of the virus of canine hepatitis in the embryonated hen's egg. The material from the twelfth egg passage was prepared in a 2 per cent suspension, filtered, and inoculated into greyhound pups which had been born and reared in isolation.

The observations of Parry, Hodgman, and Larin in England,[2] as well as those of other workers, suggested that canine hepatitis (Rubarth's Disease) resembles virus hepatitis in man in much of its ecology, but that there are considerable differences in the microscopic pathology. Sudden death with signs of circulatory collapse is common in the acute form of the canine disease, but very uncommon in man. It has been said that the disease may appear in a fatal atypical form in the neo-natal period, and, when this occurs, the characteristic intranuclear inclusions seen in the hepatic and endothelial cells of the sinusoids in other fatal cases are absent. The statement that the intranuclear inclusions are found only in fatal cases and in older animals casts some doubt on their specificity. The difficulty of being certain that stocks of dogs are free of this virus suggests that its study is of limited value unless a definitely clean stock can be studied in germ-free surroundings.

Col. Gochenour stated that if the egg-propagated canine hepatitis virus were available it would be of great interest to the manufacturers of vaccines in this country, who still use tissues of infected animals in making the killed vaccine.

Dr. MacCallum then observed that Col. Gochenour had omitted Rift Valley Fever (RVF) from his list of virus diseases of animals which might bear some resemblance to human hepatitis. RVF had first appeared among sheep in the Rift Valley of Kenya, and later in South Africa, where it affected cattle as well as sheep, and carried a high mortality rate. Until quite recently the causative agent was considered to be one of the very few "small" or "true" viruses which gave rise to systemic infection after intraperitoneal injection of the adult mouse, and which was lethal because of effects other than encephalitis. There are, of course, many other viruses which can infect newborn mice when inoculated intraperitoneally, and some, like herpes simplex, produce severe lesions in the liver. RVF virus can be found in the blood in high titers (10^{-5} to 10^{-6}) after death, and, in addition, acute focal necrosis of the liver is apparent macroscopically and microscopically, and eosinophilic inclusion bodies are found in the parenchymal cells of the liver. Like the yellow fever virus, RVF virus apparently finds essential metabolites in mouse and monkey livers. It was because of the similar proclivity for the liver exhibited by RVF and human hepatitis viruses that Dr. MacCallum had begun the studies of combinations of the two agents, to determine whether an interference phenomenon might be demonstrated.

---

[1] Nature, *168*:699, Oct. 1951.
[2] Vet. Rec. *50*:833, 1951.

Dr. Stokes asked if the horses which had been inoculated with serum hepatitis virus were ever challenged with the infectious anemia material, an experiment which Col. Gochenour had just suggested might be of possible value.

Dr. Bayne-Jones replied that the horses inoculated in the Front Royal experiment during the war had not been so challenged.

Dr. Smadel asked Col. Gochenour how it would be possible to obtain a group of horses susceptible to infectious anemia, since the disease appeared to be so ubiquitous.

Col. Gochenour replied that it would be necessary to use very young horses, and, because of the prevalence of the disease, a large group of animals—at least a dozen—would be required to ensure the probability of some positive takes after inoculation with infectious anemia material.

Dr. Syverton interjected that Dr. Charles C. Randall of Vanderbilt University, had recently demonstrated that the HeLa cell is susceptible to canine infectious abortion virus, indicating that this agent has the capacity to attack cell species other than those of its primary host.

Dr. Sabin commented that the International Committee on Virus Nomenclature and Classification had recently had to consider whether the group of pantropic viruses producing acidophilic intranuclear inclusions, and which Col. Gochenour listed in the first part of his report, ought to be categorized within a larger group of viruses producing such inclusions. This categorization would group such viruses as those of equine abortion and infectious canine hepatitis with B virus of monkeys, virus III of rabbits, and with the viruses of pseudorabies, herpes simplex and varicella, despite the differences in natural history which distinguish these viruses.

It was Dr. Sabin's opinion that such pantropic viruses as those of equine abortion and fox encephalitis do not mimic the manifestations of infectious hepatitis in man but more closely resemble agents, such as that of herpes simplex of man, which involve many tissues generally and can also produce liver lesions in newborn children. The pantropic viruses seemed to differ from the agent of human hepatitis, too, in that there was little difficulty in propagating them in tissue culture. Randall had shown earlier that equine abortion virus can grow and produce beautiful acidophilic intranuclear inclusion bodies in cultures of equine embryonic tissues, and now apparently he had grown the agent in HeLa cell cultures as well.

Dr. Smadel emphasized that one of the specific aims of the review of viruses producing hepatitis in animals was to group these agents and another use was to bring out the fact that the animal viruses producing intranuclear inclusion bodies were unlikely to be related to the agent of human hepatitis. It had been incorrectly assumed in the past that conclusions based on studies of these viruses which produced hepatitis in animals could be applied directly to the human disease.

Dr. Smadel then asked whether anyone had performed cross-neutralization tests or measured cross-immunity with the viruses of infectious abortion, or of Rift Valley Fever. The latter disease might be only a variant of the former.

Dr. MacCallum answered that he had planned to do such tests, and had also suggested doing cross-neutralization tests with Rift Valley Fever and canine hepatitis.

Dr. Werner Henle recalled that some of the early reports of Nicoleu and some of the more recent European articles had described inclusion bodies in liver biopsies taken from patients in the first few days of infectious hepatitis. Dr. Lucké's exacting study of autopsy material had not, of course, shown such inclusion bodies, but Dr. Henle wondered if there had been other studies with biopsies taken early in the course of the human disease which showed the presence of inclusion bodies.

Dr. Stokes stated that in addition to autopsy material Dr. Mallory had studied serial liver biopsies and had not observed inclusion bodies.

Dr. MacCallum added that a study of serial liver biopsies made at the Post Graduate Hospital in London also had not revealed inclusion bodies.

Dr. Smadel then asked whether any attempts had ever been made to study the possible relationship between the intranuclear inclusion pneumonia of children and the comparable disease in dogs.

Dr. Werner Henle answered that an attempt has been under way in Philadelphia to discover a relationship between the human and canine diseases.

Dr. Sabin added that the pulmonary disease in children is probably related to the salivary gland virus, which produces inclusion bodies, and that Dr. Margaret Smith of St. Louis had recently succeeded in propagating human salivary gland virus in human tissue culture. This virus had now been successfully carried through five passages, and should prove valuable in correlating the disease with the similar disease seen in dogs.

Dr. Paul then asked Dr. Havens to present his review of attempts to test for immune bodies against the hepatitis agent.

IV. REVIEW OF ATTEMPTS TO TEST FOR IMMUNE BODIES PRODUCED AGAINST THE HEPATITIS AGENT

DR. W. PAUL HAVENS, JR.

ATTEMPTS BY OTHERS

The occurrence of a number of nonspecific and apparently different serologic reactions in patients with viral hepatitis has been described. Precipitins and complement-fixing antibodies have been demonstrated in convalescent phase sera which react with substances in acute phase sera and in saline extracts of normal human liver and liver from patients with hepatitis (1, 2). The agglutination of certain strains of Salmonella (3-7)

and sheep erythrocytes (2, 8), and the development of transient, falsely positive Wassermann and Kahn reactions (9, 10) in the sera of patients in the convalescent phase of disease, have been recorded. However, the frequency of their occurrence has varied widely, and certain workers (11-17) have failed to substantiate the high percentage of positive results reported by others. Evans (18) reported that 26 and 32 per cent of sera from two groups of patients with hepatitis agglutinated human erythrocytes modified by two strains of Newcastle disease virus. The significance of this reaction is undetermined, although it is of particular interest in view of other similarities between hepatitis and infectious mononucleosis, since a goodly percentage of sera from patients with the latter disease also agglutinate similarly treated human erythrocytes.

Early attempts by Gear (1) to devise a specific diagnostic test in serum hepatitis resulted in the demonstration of a precipitin and complement-fixing antibody in convalescent serum which reacted with an antigen found in acute phase serum. An analogy was made between this serum antigen-antibody system and a similar reaction between acute and convalescent phase sera in yellow fever described by Hughes (19), who originally suggested that the antibody elaborated in convalescence was a response to an antigenic substance resulting from injury to the liver and unrelated to the causative virus. There is no reason to believe that this might not also be true in serum hepatitis, and actually there are certain manifestations in patients with this disease (arthralgia, urticaria) which suggest the possibility of such an immunologic reaction. In support of this was the observation that the antigen and antibody found in sera from patients with serum hepatitis gave precipitin reactions when mixed with the Hughes antibody and antigen derived from the sera of monkeys infected with yellow fever. In addition, the antibody in the sera of monkeys convalescent from yellow fever and in the sera of patients convalescent from serum hepatitis gave positive precipitin reactions with antigens made from extracts of liver from normal monkeys and a number of small laboratory animals. The occurrence of this serum antigen-antibody reaction has been demonstrated by others (20), utilizing sera obtained from patients with infectious hepatitis as well as serum hepatitis, and recently Pollard and Bussell (21) described a substance in the acute phase serum of a patient with serum hepatitis which fixed complement with sera from patients recovering from infectious hepatitis or with other forms of jaundice. Such specificity has not been found, however, by others.

Eaton *et al.* (2) suggested that the serum antigen-antibody system is distinct from another reaction in which a heterogenetic antibody, which may be absorbed by human liver or sheep cells, is concerned. This antibody agglutinates sheep erythrocytes and fixes complement with antigen made from saline extracts of liver from fatal cases of hepatitis and also from normal liver. Although the agglutinating and complement-fixing properties were believed to belong to a single antibody system, there was no consistent relationship between the two. It may be differentiated from

other heterogenetic antibodies primarily by its absorption by human liver. It is apparently different from the heterophile antibody found in infectious mononucleosis and atypical pneumonia, and it is not related to Forssman's antigen, to the heterogenetic antigen common to human blood group A substance, or to the Wassermann reagin. It occurred in both acute and convalescent phases of hepatitis, but was also found in a smaller percentage of patients with atypical pneumonia, and in normal persons. Similar complement-fixing antibodies were observed by Bjørneboe and Krag (22) and by Miles (23). In addition, Findlay *et al.* (24) found positive reactions to antigens made from infected liver but not from normal human liver, which led them to interpret their positive results in yellow-fever-vaccine hepatitis, epidemic hepatitis, and post-arsphenamine hepatitis as evidence of specific reactions reflecting an antigenic relationship among these three conditions. This was not borne out by the work of others. Of interest, however, in this regard were the results of Miles (23), who postulated two different and separate serologic reactions: (a) an organ-specific reaction with normal or infected liver, and (b) a specific reaction between an antigen made from the liver of fatal cases of post-arsphenamine hepatitis and sera from patients with this disease. In contrast to the organ-specific reaction, the latter complement-fixing antibody more frequently appeared late in disease, usually after eight weeks, and there were no cross-reactions between antigens and sera of this reactive system and materials obtained from patients with epidemic hepatitis or other forms of serum hepatitis. Miles (23) described a high percentage of positive reactions occuring in the sera of one group of patients who had post-arsphenamine jaundice during an epidemic in a military clinic, while another group of patients subsequently tested had consistently negative results. This was interpreted to indicate a specific reaction related to an agent which was widespread at the time and which apparently became less common.

Precipitins were also found in the sera of patients with epidemic hepatitis which reacted with antigens made from liver and spleen. Lubling and Olitzki (25) isolated from the livers of cattle an alkali-stable preparation, thought to be largely glycogen, which reacted with sera from patients with hepatitis as well as other diseases in which hepatic damage occurred, i.e., brucellosis, malaria, and typhus. Similar results were also obtained by Kligler and Bernkopf (26), using an antigen made from the spleen of a patient with epidemic hepatitis. Olitzki and Bernkopf (27), using a cholesterolized alcoholic extract of liver or spleen, found precipitins in the blood of 94 out of 102 patients with hepatitis, and in 64 out of 330 patients with other diseases. The positive tests in patients with hepatitis occurred as early as three days after onset of disease. These reactions were independent of positive Kahn or cephalin flocculation reactions. Heating to 60° C. destroyed most of the positively reacting substances in the sera from patients with diseases other than hepatitis; in addition, 86 per cent of the hepatitis sera were positive in tests using 0.02 ml. of serum or less, while at least 0.1 ml. of serum from patients with other diseases was required for a positive test.

Henle and his associates (28) used amniotic fluid presumably containing inactivated hepatitis virus A for making skin tests, and found a high percentage of positive tests in patients recovered from infectious hepatitis. Unfortunately, the technical difficulties involved have made it impossible to obtain consistently reproducible results, and these observations await confirmation by others. Numerous attempts to prepare effective antigens were also made by Henle (29), who concentrated materials from presumably infected tissue cultures or embryonated eggs. These efforts were unsuccessful, and all such materials were either anti-complementary or nonreactive.

ATTEMPTS IN AUTHOR'S LABORATORY

A number of attempts have been made in this laboratory to devise a serologic test for viral hepatitis. Convalescent serum has yielded a complement-fixing antibody which reacts, as previously described by others, with acute phase serum and extracts of human liver. In addition, unsuccessful attempts were made to obtain an antigen for use in precipitin tests by the concentration of urine from patients in the acute phase of disease.

An inherent difficulty in the development of a specific serologic test has been the inability to obtain an adequate amount of material containing sufficient virus to be demonstrably reactive in vitro. Since virus is known to be present in the feces of patients during the acute phase of infectious hepatitis, an effort was made to purify and concentrate virus from such material by precipitation with methanol at low temperatures and ultracentrifugation (30). A number of antigens were made using pools of feces obtained from patients with hepatitis and also from normal persons. Using human volunteers, Dr. Joseph Stokes, Jr., made a single attempt to demonstrate infectivity of an antigen prepared from presumably infectious stools, but this was not successful. Although this antigen and other similar ones made from feces of patients with hepatitis, and also of *normal* persons, had the capacity to fix complement in the presence of 82.5 per cent of sera of a group of patients with viral hepatitis and with only 20 per cent of sera of normal persons, positive reactions were also found in 50 per cent of patients with hepatic cirrhosis and in a smaller percentage of patients with atypical pneumonia and positive Wasserman reactions. No definite qualitative trend of antibody response was observed in relation to the phase of hepatitis, although a somewhat greater percentage of positives occurred from the third to the seventh week of disease. The antigen concerned in this reaction was stable at a temperature of 60° C. for 30 minutes and insoluble in alcohol, ether, and benzene. The antibody was not absorbed by human liver, boiled guinea pig kidney, or by beef, sheep, or rabbit erythrocytes. Although its exact nature was undetermined, it was thought to be heterogenetic.

Because of the apparent failure to produce a specific antigen from the feces of patients with hepatitis, an effort was made to develop a method which would detect virus in the sera of patients in the acute

phase of disease. The capacity of collodion particles to enhance the precipitin reaction by agglutination in the presence of an antigen-antibody system was utilized (31). Gamma globulin prepared from the sera of patients convalescent from hepatitis* and normal human gamma globulin were used as sources of antibody. Acute phase sera obtained during the first three weeks of hepatitis were used as "antigen". For purpose of control, sera ("antigens") obtained from normal donors, patients with positive Wasserman tests, and patients with a variety of infectious and metabolic diseases were used. In addition, serum obtained in the convalescent phase of hepatitis was tested for reactivity as "antigens". The tests were performed by sensitizing the collodion particles to varying dilutions of "antigen" for two hours at room temperature before mixing with the antibody. Collodion particles sensitized by acute phase sera were agglutinated in a 73.5 per cent of tests when mixed with either sources of antibody. However, the sera from patients with other acute infectious diseases gave positive reactions in 56.7 per cent of tests, although the titer was, in general, lower than in the sera of patients with hepatitis. In contrast, only a small percentage of sera from patients with various degenerative diseases or positive Wasserman tests, or from normal persons, gave positive reactions.

The nature of the reacting substances is not clear. Heating the antibody (gamma globulin) at 56° C. for 30 minutes did not affect its capacity to agglutinate collodion particles sensitized with acute phase serum. Heating the acute phase serum to 50° C. did not affect the agglutination, although heating at 56° C. for 30 minutes inhibited it. The substance in acute phase serum was soluble in benzene, ether, and acetone. Incubation of acute phase sera and antibody with sheep or rabbit erythrocytes, human group 0 cells, or boiled bovine erythrocytes did not affect the reaction, while incubation with boiled guinea pig kidney altered it somewhat, and incubation with human liver nullified it. The substance in acute phase serum which reacted with collodion particles in such a way that they were agglutinated in the presence of gamma globulin was associated with the globulin fraction, and albumin obtained from fractionated acute phase serum was completely inactive. The solubility of the reactive substance in lipid solvents and its association with the globulin fraction of the serum suggest that it is lipoprotein. This is consistent with the known decrease of albumin and increase in globulins, particularly with alpha and beta components, in the acute phase of viral hepatitis as well as in a number of other unrelated infectious diseases.

It is not likely that virus is an integral part of this serologic reaction, in which apparently similar positive tests occurred in patients with other acute infectious diseases. The fact that the sources of both the normal and the convalescent hepatitis globulin were adults suggests the probability that a wide spectrum of antibodies was present which might react with a number of different antigens, thereby obscuring a specific

* The fractionation of the convalescent phase hepatitis sera was done by E. R. Squibb and Sons, New Brunswick, N. J., through the courtesy of Dr. John W. Palmer.

reaction. Unfortunately, the lack of significant differences in the degree of reaction between the globulin obtained from normal persons and patients convalescent from hepatitis made it impossible to ascribe any specificity to the positive tests. It is of interest, however, that the maximum positive titer of "antigen" recorded for other infectious diseases was 1:8, while almost half of the sera from patients with viral hepatitis had titers greater than this.

The fact that exposure of either the reactive acute phase sera or the gamma globulin to human liver in many instances nullified the reaction need not imply any organ-specific significance, although further clarification of this point is necessary. It is possible that the suppressive action of human liver on the reacting substances is a physicochemical one, unrelated to any antigen-antibody system.

The interpretation of previous demonstrations of antigenic substances in acute phase hepatitis and yellow fever sera which react with precipitins and complement-fixing antibodies in convalescent phase sera has been based on the concept of an antibody developing during convalescence in response to an antigenic substance resulting from injury to tissue and unrelated to the causative viruses. It is possible that the reaction described here is again representative of this phenomenon.

SUMMARY

A number of serologic reactions have been described in viral hepatitis; most of them have been accepted as nonspecific. In those for which claims of specificity have been made, the attempts of others to repeat them have been unsuccessful. The lack of a susceptible laboratory animal and the apparent failure to propagate hepatitis virus in embryonated eggs or tissue culture have impeded progress along this line.

References

1. Sawyer, W. A., Meyer, K. F., Eaton, M. D., Bauer, J. H., Putnam, P., and Schwentker, F. F.: Jaundice in Army personnel in the western region of the United States and its relation to vaccination against yellow fever, Part I—Am. J. Hyg. *39*:337-430, May 1944; Parts II, III, and IV—Am. J. Hyg. *40*:35-107, July 1944.
2. Eaton, M. D., Murphy, W. D., and Hanford, V. L.: Heterogenetic antibodies in acute hepatitis, J. Exper. Med. *79*:539-557, May 1944.
3. Sarrailhé, A., and Clunet, J.: La "jaunisse des camps" et l'épidémie de paratyphoide des Dardanelles, Bull. et mém. Soc. Méd. d. hôp. de Paris, 3rd series, *40*:45-60, 1916.
4. Cantacuzene, J.: Sur une épidémie d'ictère observée en Roumanie pendant la campagna de 1917, La Presse Médicale, *26*:541-543, 1918.
5. Anigstein, L., and Milinska, Z.. Investigations on jaundice of bacterial origin, J. Trop. Med. *26*:337-340, 1923.
6. Nichols, H. J.: Agglutination of typhoid group of organisms in cases of jaundice among vaccinated persons, J.A.M.A. *81*:1946-1948, 1923.
7. Havens, W. P., Jr., and Wenner, H. A.: Infectious hepatitis complicated by secondary invasion with Salmonella, J. Clin. Invest. *25*:45-52, Jan. 1946.
8. Leibowitz, S.: Heterophile antibody in normal adults and in patients with virus hepatitis, Am. J. Clin. Path. *21*:201-211, March 1951.

9. Kuzell, W. C., and Puccinelli, V.: False positive serology in infectious hepatitis, M. Bull. North African Theat. Op. (No. 3) *2*:57-59, Sept. 1944.
10. Waelsch, J. H.: Transient non-specific Wassermann and Kahn reactions in case of infective hepatitis, Brit. M. J. *1*:353-354, 9 March 1946.
11. Fox, J. P., Manso, C., Penna, H. A., and Pará, M.: Observations on the occurrence of icterus in Brazil following vaccination against yellow fever, Am. J. Hyg. *36*:68-116, July 1942.
12. Oliphant, J. W., Gilliam, A. G., and Larson, C. L.: Jaundice following administration of human serum, Pub. Health Rep. *58*:1233-1242, 13 Aug. 1943.
13. Havens, W. P., Jr., Gambescia, J. M., and Knowlton, M.: Results of heterophile antibody agglutination and Kahn tests in patients with viral hepatitis, Proc. Soc. Exper. Biol. & Med. *67*:437-440, April 1948.
14. Barker, M. H., Capps, R. B., and Allen, F. W.: Acute infectious hepatitis in the Mediterranean Theatre, J.A.M.A. *128*:997-1003, 4 Aug. 1945.
15. Zimmerman, H. J., Lowry, C. F., Uyeyama, K., and Reiser, R.: Infectious hepatitis; clinical and laboratory features of 295 cases, Am. J. M. Sc. *213*:395-409, April 1947.
16. Berk, J. E., Shay, H., Ritter, J. A., and Siplet, H.: Infectious mononucleosis and infectious hepatitis; studies bearing on certain resemblances and differences, Gastroenterology *11*:658-671, Nov. 1948.
17. Zimmerman, H. J., and Thomas, L. J.: Anicteric hepatitis: a report of 9 sporadic cases, Am. J. M. Sc. *216*:545-550, Nov. 1948.
18. Evans, A. S.: Serological studies on infectious mononucleosis and viral hepatitis with human erythrocytes modified by different strains of Newcastle disease virus, J. Immunol. *64*:411-420, May 1950.
19. Hughes, T. P.: Precipitin reaction in yellow fever, J. Immunol. *25*:275-294, Sept. 1933.
20. Havens, W. P., Jr.: Unpublished results.
21. Pollard, M., and Bussell, R. H.: Rapid laboratory diagnostic procedures in microbiology: complement-fixation reactions in serum hepatitis, USAF School of Aviation Medicine, Project No. 21-1401-0005, Report No. 3, June 1953.
22. Bjørneboe, M., and Krag, P.: Studies on a complement-fixation in hepatitis with liver extract as the antigen, Acta path. et microbiol. scandinav. *24*:352-361, 1947.
23. Miles, J. A. R.: Serological investigation in hepatitis using the complement-fixation reaction. Brit. J. Exper. Path. *27*:25-33, Feb. 1946.
24. Findlay, G. M., Martin, N. H., and Mitchell, J. B.: Hepatitis after yellow fever inoculation; relation to infective hepatitis, Lancet *2*:301-307, 2 Sept. 1944; 340-344, 9 Sept. 1944; 365-370, 16 Sept. 1944.
25. Lubling, P., and Olitzki, L.: Antigenic properties of glycogen, Harefuah *22*:26, 15 Jan. 1942.
26. Kligler, I. J., and Bernkopf, H.: Cited by Olitzki, L., and Bernkopf, H. (See reference No. 27).
27. Olitzki, L., and Bernkopf, H.: A precipitation test in infective hepatitis, J. Inf. Dis. 60-67, July-Aug. 1945.
28. Henle, G., Drake, M., Henle, W., and Stokes, J., Jr.: A skin test for infectious hepatitis, Proc. Soc. Exper. Biol. & Med. *73*:603-605, April 1950.
29. Henle, W.: Personal communication.
30. Havens, W. P., Jr., Lloyd, H., Melnick, J. L., and Colbert, J. W., Jr.: Complement-fixation with fecal antigens in viral hepatitis, J. Inf. Dis. *85*:139-145, Sept.-Oct. 1949.
31. Havens, W. P., Jr., and Eichman, P. L.: Collodion particle agglutination with acute phase sera and immune globulin in viral hepatitis, J. Immunol. *64*:349-356 April 1950.

DISCUSSION

Dr. Morris asked whether anyone had tried to determine whether complement-fixing antibodies were present in the supernatant fluid of the tissue cultures in which attempts had been made to propagate human hepatitis virus.

Dr. Havens answered that Dr. Werner Henle had made such determinations, and asked him to comment.

Dr. Werner Henle said that the supernatants were non-reactive or gave non-specific reactions with his samples of stored sera obtained from volunteers before inoculation and as long as six months after inoculation. The reactions with pre- and post-inoculation sera were often equal.

Dr. Enders asked whether tests had been made for antigens in the fluid from human cell cultures to which material containing hepatitis virus had been added.

Dr. Gertrude Henle replied that such tests had been made, but the fluid was found to be anticomplementary.

Dr. Evans commented that his study of the reaction between red cells modified by Newcastle virus and sera from patients with infectious hepatitis had been extended to include 300 cases of hepatitis. Although the incidence of positive agglutination tests with hepatitis sera was over 20 per cent, far higher than with control sera, there was no rise in agglutinin titer with convalescence. The reaction was an all-or-none affair, since it was either positive or negative throughout the course of the disease.

Dr. Werner Henle added that his experience with red cells modified by Newcastle virus had been similarly disappointing.

Dr. MacCallum elaborated upon Dr. Havens' references to the work of Drs. Findlay and Miles on antibodies appearing in the sera of patients convalescent from hepatitis. Dr. Findlay had decided after further study that the antibody which he had described was not specific. Dr. Miles had later developed similar doubts as to the specificity of the complement-fixing antibody he had described in post-arsphenamine hepatitis. He was unable to obtain an antigen from the livers of patients with post-arsphenamine hepatitis which would give results as encouraging as those obtained with the first antigen he had employed.

Dr. Paul then asked Dr. Havens for his opinion of a recent German report which claimed that 60 per cent of cases of infectious hepatitis have elevated cold agglutinin titers.

Dr. Havens replied that while he was in Germany he had measured cold agglutinins in the blood of patients with hepatitis and had been able to demonstrate elevated titers in only a very small percentage.

Dr. Paul next asked Dr. Havens whether it was necessary to be so deeply concerned about positive cross-reactions between supposed hepatitis antigens and sera from patients with other diseases. If a complement-fixing reaction for hepatitis behaved like those of mumps, one would

expect to find some positive reactions even in normal controls as evidence of past infection.

Dr. Havens answered that, unfortunately, he had insufficient amounts of the post-inoculation sera he had studied, and no pre-inoculation sera, to allow more careful evaluation of the specificity of the reaction in hepatitis. In addition, it had been discouraging to find antigens in normal feces which proved to be as effective as those from the feces of patients with hepatitis.

Dr. Evans added that, like Dr. Havens, he had been unable to produce an antigen by concentrating urine from patients with acute hepatitis. Agglutination tests with sensitized cells were tried, as well as precipitin tests, but no specific results were obtained.

V. SOURCE, POTENCY, AND AVAILABILITY OF CLINICAL MATERIAL FOR LABORATORY TESTING

DR. RODERICK R. MURRAY

During the course of our studies on the safety of blood and blood products with respect to serum hepatitis, a number of samples of serum and of plasma have been tested for infectivity by inoculation into human volunteers. Some of these were obtained in the course of studies which were carried out jointly with Dr. Neefe and his co-workers on the problem of "carriers" of hepatitis. Others were studied in the course of obtaining a large pool of known icterogenic material which could be used in most of the studies on the sterilization of plasma and plasma products. This pool has been referred to as Infected Pool Plasma and is designated here by the code NIH-8, which was given to the frozen material after the preparation of the pool. A few of the cases studied represent second passage material from subjects inoculated with NIH-8.

Table I, taken from a recent paper on the carrier problem (1), shows the materials tested and the cases which resulted in the course of this particular study. Sera were collected from each of the volunteers at weekly intervals, or more frequently if they were hospitalized. Portions of these sera have been maintained in the frozen state and are available for further study. In addition, larger bleedings are available from the second group of volunteers inoculated with serum from donor L.H. and the second group inoculated on the same date with serum No. 1 from H. H. These bloods were collected every two weeks, and covered the latter part of the incubation period and the earlier part of the acute illness.

Table II shows the complete list of materials which have been proved to be infectious by inoculation into volunteers. Materials designated M. M., L-125, and L-154 represent second passage material from NIH-8 in the sense that subjects M. M., L-125 and L-154 developed hepatitis as a result of being inoculated with NIH-8. Similarly, L-179 + L-181 repre-

TABLE I

RESULTS OF INOCULATION OF VOLUNTEERS WITH SERUM FROM SUSPECTED DONORS

Case	Donor	Days Since Incriminated Donation	No. Volunteers Inoc.	Cases of Hepatitis			Incubation Period (Days)	
				Jaundice		Total	In Volunteers[c]	In Original Recipient
				With	Without			
1	W.C.	203	10	0	0	0	——	65
2	L.H.	385	10 5	4 0	2[a] 1	6 1	(18),30,45,46,(46),56 (43)	48
3	V.S.	356	10	1	0[a]	1	48	40
5	R.F.	149	10	1	0[a]	1	84	70
7	H.H.	135	10 5	4 5	0 —	4 5	35,50,56,72 43,49,56,57,63	44
8	C.D.	43	10	2	1[b]	3	(50),57,67	32

[a] One additional subject in each of these groups showed development of equivocal or abnormal hepatic tests suggestive of hepatitis without jaundice.

[b] Three additional subjects in each of these groups showed development of equivocal or abnormal hepatic tests suggestive of hepatitis without jaundice.

[c] Incubation periods in parentheses refer to cases of hepatitis without jaundice.

TABLE II

MATERIALS HAVING INFECTIVITY PROVED BY INOCULATION INTO VOLUNTEERS

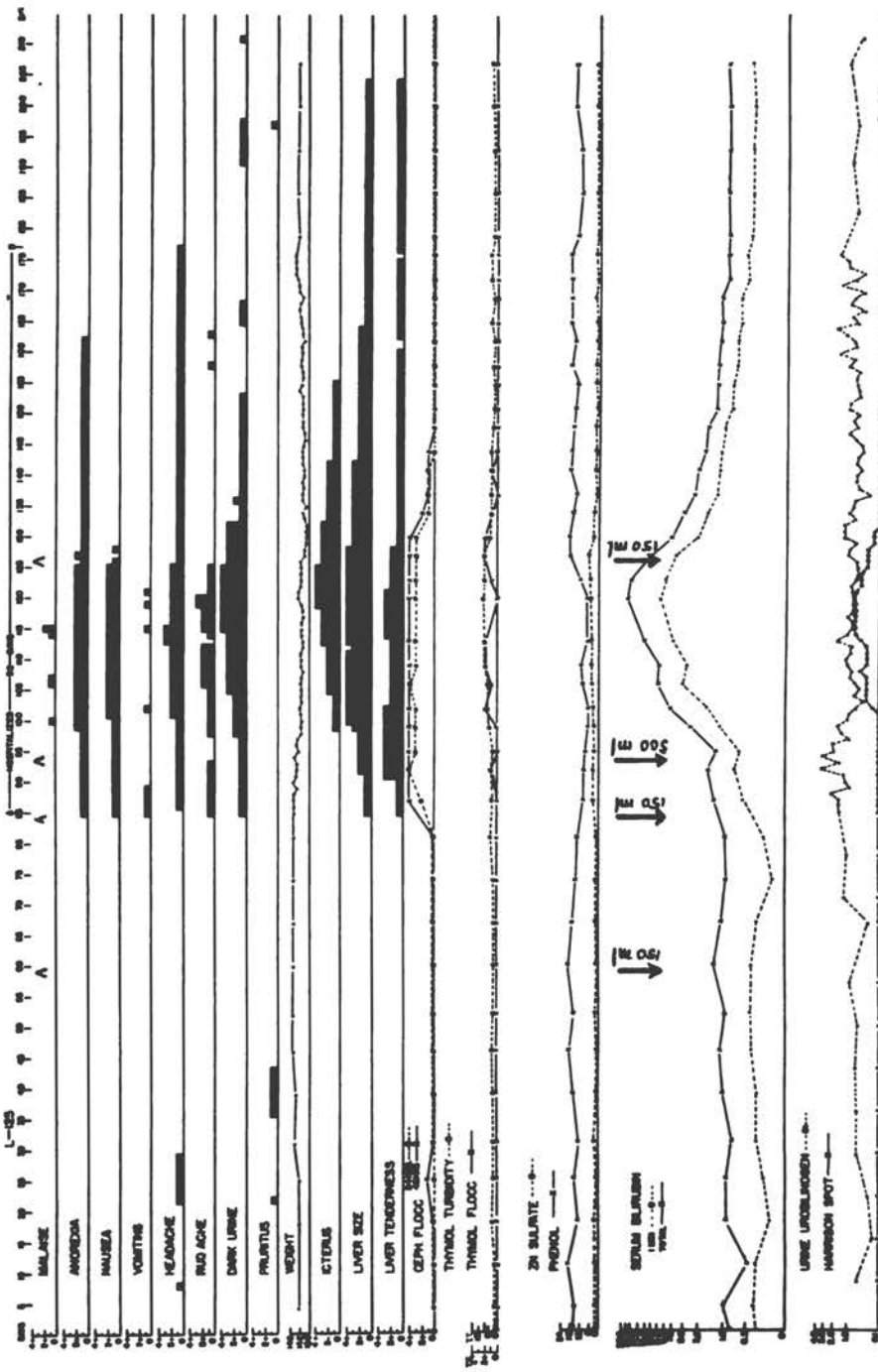
	Infectivity	Incubation (Days)	Amount Remaining	Attempts at Propagation
R.R.	3/8	54-81	10 ml.	
NIH-8 (Frozen)	52% ∓	45-125	2.5 L.	+ *
NIH-8 (Dried)	4/10	62-96	Large Amount	
L.H.	7/15	18-56	0	
H.H. No. 1	9/15	35-72	10 ml.	*
H.H. No. 2	2/5	44-46	15 ml.	+ *
R.F.	1/10	84	12 ml.	
V.S.	1/10	48	12 ml.	
C.D.	3/10	50-67	0	
Thrombin (G-6058)	6/10	73-101	21 pkgs.	
W.P.R.	3/6	75-104	10 ml.	+
M.M.	1/5	91	200 ml.	
L-179+L-181	1/6	109	200 & 200 ml.	+
L-125 (Whole blood)	1/5,2/10	48,70-77	120 ml.	+
L-154	1/3	75	60 ml.	
Rollson (Stool)	3/6	22-31	0	+

\+ Direct attempts at propagation.
* Attempts at propagation from material derived from subjects inoculated with these materials.
∓ Infectivity of this material proved by titering in volunteers was 1.0×10^{-4} ml.

sents second passage material from H. H. No. 1. These samples of plasma were pooled for the purpose of the study recorded in table II, but are available separately.

Special mention should be made of H. H. Nos. 1 and 2. No. 1 represents serum from a suspected carrier, H. H., who had been implicated as the source of hepatitis which had occurred in the recipient of blood which he had donated. This sample of serum was drawn 135 days after this blood donation and caused hepatitis in nine out of 15 volunteers who were inoculated. The donor later developed hepatitis with jaundice himself, and was bled again approximately six months after recovery. This serum is designated H. H. No. 2. This was inoculated into five volunteers. Two of these developed hepatitis. Both lapsed into hepatic coma, and one died.

In all, 145 cases of hepatitis have developed following inoculation of the above materials or of products such as albumin, ultraviolet-treated plasma, etc., derived from them. The illnesses encountered were of all degrees of severity. Subjects remained on the program from five to six months, and blood was collected at weekly intervals. In the case of hospitalized subjects and those under close observation, bloods were collected at more frequent intervals. All of these sera have been stored and are available for study. In addition, as indicated in the preceding paragraph, larger bleedings, usually 150 ml., were made in some instances. Blood was collected in ACD solution in pediatric bleeding bottles. A typical example is shown in the accompanying chart. This shows the course of



Legend for Chart: Course of illness in subject L-125, with hepatitis following inoculation of infected pool plasma NIH-8.

illness in a subject designated L-125 who developed hepatitis as a result of the inoculation of NIH-8. Weekly or more frequent bleedings were taken for hepatic function tests, and the serum in each case has been retained. In addition, 150-ml. amounts of blood were taken at the times indicated by arrows. These latter bleedings straddle what is probably the period of highest infectivity. Similar series of bleedings are available from a number of other individuals who have been inoculated with NIH-8: L. H., H. H. Nos. 1 and 2, L-179 + L-181, L-125, and L-154. Some of these developed hepatitis, while others showed no clinical or laboratory changes.

The ability to transmit hepatitis depends to some extent on the stage of the illness. From what few studies have been made with this in mind, it appears that the infectivity may actually be at a maximum before the onset of jaundice, and decline as the illness progresses. Viremia may, however, be persistent, as was illustrated in the case of M. M., whose serum is listed in table II. This individual had developed hepatitis as a result of inoculation of NIH-8. He had recovered from his illness and was four months convalescent at the time the sample in question was drawn. His liver function tests at this time were normal.

The serum designated W. P. R. in the above table is of considerable interest. This was obtained from a case which occurred in a military establishment at a time when infectious hepatitis was prevalent. A diagnosis of infectious hepatitis had been made in the case of W. P. R., and when the serum was administered to volunteers it was expected that hepatitis of short incubation period would result. The incubation period, however, varied from 75 to 104 days. This experience is similar to that reported recently by Evans (2).

A considerable number of samples of plasma, separated from full-sized blood donations (480 ml.), are available from a number of individuals who developed hepatitis in the course of these studies. These bloods were taken at various times during convalescence (six months to two years), and were obtained because the individuals concerned presented themselves as blood donors. The bloods were rejected because of the fact that the donors had participated in the hepatitis program, but rather than being discarded were turned over to the National Institutes of Health for possible future study.

References

1. Murray, R., Diefenbach, W. C. L., Ratner, F., Leone, N. C., and Oliphant, J. W.: J.A.M.A. *154*:1072-1074, 27 March 1954
2. Evans, A. S.: Hepatitis in U. S. troops in Germany, Proc. Soc. Exper. Biol. & Med. *75*:809-811, Dec. 1950.

DISCUSSION

Dr. Smadel suggested that since persons concerned with the hepatitis problem were now working together more closely, that in the future they might keep Dr. Murray informed as to the results of studies involving the materials which he supplied to them. In this way information on

the infective titers of available material known to contain the viruses of SH or IH would be conveniently centered in one place.

Dr. Tillett added that one of the principal reasons for having asked Dr. Murray to present his excellent review of materials of known infectivity was to point out the great advantage of a central agency where well-documented infective material would be available for use in further study. Such centralization would simplify and standardize studies of human hepatitis and obviate the errors which could arise when each investigator used only his own isolated samples.

Dr. Syverton asked Dr. Murray to explain what position was occupied in his table by the sera designated L-204, L-207, and "Merriam", which Dr. Syverton had received for inoculation into his antibody-free swine.

Dr. Murray explained that the materials listed on his table represented only those which had been proved by human inoculation to be infectious. Sera which had been designated by other numbers, and which had been distributed to Dr. Syverton and others for study, represented second-passage material from the proved inocula. L-204 and L-207, for example, were sera taken from cases of hepatitis which developed after inoculation with HH 2. "Merriam" plasma was identical with the material designated in the table as L-125. The donor of this plasma had an incubation period of approximately 85 days after inoculation with NIH-8. Inoculation of his acute-stage whole blood (as the control portion of a study of the efficacy of beta propiolactone as a sterilizing agent) produced hepatitis in two of five volunteers. These volunteers had relatively short incubation periods, and were seriously ill.

Dr. Stokes asked whether Dr. Murray had preserved stool specimens from any of his volunteers with homologous serum hepatitis.

Dr. Murray replied that he had done so, but none of these stool specimens had been tested for infectivity.

Dr. Stokes wondered whether the liver function tests of the volunteer designated as "M. M." had returned to normal four months after recovery from the initial illness produced by NIH-8 plasma, when his serum had again been proved to be infective.

Dr. Murray said that the liver function tests of "M. M." were again normal four months after recovery, at which time he was proved to have a persistent viremia. No liver biopsy had been obtained from this man.

Dr. Smadel asked whether Dr. Murray had any material on hand from cases of infectious hepatitis (IH) which was of proved infectivity.

Dr. Murray replied that he did not.

Dr. Mirick asked whether Dr. Werner Henle still had any of the original Akiba plasma.

Dr. Werner Henle replied that only a few small samples of this material remained available, and it was not definitely established which of the samples was infective.

He added that a problem existed with materials suspected of containing IH, in that it was considered inadvisable to pool them. This was

because it was not quite certain at what point in the disease immune antibodies arise, and materials containing such antibodies might be added inadvertently during the constitution of an infectious pool. For example, Dr. Gordon had been able to produce hepatitis by administering samples from an initial small pool of Akiba material in dilutions of 1:100 and 1:400, and yet in later studies with material from another pool, Dr. Henle had been unable to produce cases with dilutions of 10^{-1} to 10^{-4}. It was not until each of the eight samples contributing to the last pool were fed separately to volunteers that cases of hepatitis could be produced. This suggested that one of the samples in the pool Dr. Henle had used had contained sufficient immune antibody to neutralize the virus present in other samples.

Dr. Smadel asked whether samples of plasma were available from any of this last group of individuals who did become ill with hepatitis after being fed Akiba plasma.

Dr. Werner Henle replied that many small samples of plasma from volunteers inoculated with Akiba material by Dr. Mirick and himself were available. However, the total volume was very small, and the samples had not been individually tested for infectivity.

Dr. Snyder asked whether it was known at what temperature the immune antibodies for IH virus could be destroyed by heat, and whether any differential existed between the amount of heat which would destroy the antibodies and the amount which would kill the virus.

Dr. Paul replied that the antibody against infectious hepatitis virus was, as yet, poorly defined.

Dr. Murray added that it was known that infected plasma heated for four hours at 60° C. still produced hepatitis, although the gamma globulin in the plasma was denatured by this amount of heat.

Dr. Paul then asked whether anyone present had a supply of proved infective material available in addition to those already mentioned.

Dr. Gertrude Henle stated that one liter of Fort Bragg serum which had been proved infective was still stored at Princeton.

Dr. Sabin noted that he still had one batch of frozen and lyophilized serum from cases of sandfly fever, which had produced hepatitis in inoculated volunteers. Another batch of serum was also available which had also been obtained from a patient with sandfly fever and which had produced hepatitis in two of three recipients.

Dr. Sabin then asked Dr. Murray whether any of the materials listed on his chart could be considered, because of short incubation periods, to contain IH virus.

Dr. Murray replied that he did not feel that any of the materials listed could be considered to contain IH virus. The shortest incubation period listed was 18 days, occurring in one of the recipients of serum from L. H. Although none of L. H.'s serum remained, sera had been obtained and stored from those seven patients who had developed hepatitis fol-

lowing inoculation of serum from L. H. (The mean of their incubation periods was 40 days.)

Dr. Enders stated that he had a store of feces and serum collected early in the disease from five to six patients who were studied during an epidemic of typical infectious hepatitis in an institution. Material from one of these patients had been tested for infectivity by Dr. Murray.

Dr. Murray said that the single sample he had received from Dr. Enders had not produced hepatitis. However, the other samples from this group might be infective.

Dr. Evans asked if it had been determined how long material stored in the frozen state or at room temperature remained infective.

Dr. Murray stated that the oldest frozen material which he presently had available was NIH-8, the pool which had been constituted originally in 1951. Derivatives of this material had produced hepatitis after inoculation as recently as several months previous to this Conference. In one experiment, NIH-8 plasma had been stored at room temperature in the liquid state. After three months of storage, the plasma produced hepatitis in three of five recipients; after six months, it produced hepatitis in only one of 19 recipients, and this single case occurred after a protracted incubation period of 194 days.

VI. GENERAL DISCUSSION

Dr. Paul then called upon the participants to conclude the Conference with a general discussion of the subject. Since Dr. Dalldorf was one of those who had originally helped organize the meeting, Dr. Paul asked him to be the first to comment.

Dr. Dalldorf emphasized that there still seemed to be good evidence from the histology of the lesions of hepatitis, particularly on the basis of the abnormal cells described by Dr. Tracy Mallory, to imply that the pathologic changes had been directly caused by a virus. The significance of such specific changes in hepatitis might prove to be as great as that of the abnormal or giant nucleus in measles, or of inclusion bodies which actually represent intracellular virus colonies. It was becoming clearer, with electron microscope studies, that the specific cellular changes due to viruses could be separated from changes resulting from death of the cell or the action of an extrinsic toxin.

Dr. Smadel indicated that several of the topics discussed had been of particular interest to him. Dr. Bang's presentation had carefully enumerated the many complex factors which must be considered in tissue culture work. The increasing interest of tissue culture experts in the problem of cultivating the hepatitis virus should prove invaluable. As suggested by Drs. Gey and Bang, attempts should be made to centrifuge the virus of hepatitis directly onto living tissue culture cells. Preliminary evidence suggested that small amounts of virus which could not be otherwise detected were picked up by the use of direct centrifugation onto cells, and the method might prove fruitful in studies of both hemorrhagic fever and hepatitis.

Another suggestion recently made by Dr. Gey in relation to hemorrhagic fever might also be applied to hepatitis. After discussion with geneticists and experts in tissue transplantation, he had developed the idea that heterozygous tissue transplants might be made to take if embryos were inoculated with the transplants in utero. The technique might not be as successful with transplants of human tissue to the guinea pigs as it had been with the livers of inbred mice. However, Dr. Smadel planned to utilize it in studies of hemorrhagic fever, inoculating guinea pigs in utero with human tissues and then inoculating them with infectious material after they were born.

Dr. MacCallum had made the pertinent suggestion that if monkeys were employed in transmission studies, fairly large groups of animals must be used. The large colonies used in poliomyelitis studies were still available, and might make it feasible for investigators interested in hepatitis to utilize adequate groups of monkeys.

Another idea which seemed worth pursuing further was the possible relationship between infectious anemia of horses and serum hepatitis, to which Col. Gochenour had alluded.

Dr. Dalldorf felt that if any direct relationship existed between human serum hepatitis and infectious anemia of horses, it would have become evident long ago in the course of the widespread administration to humans of diphtheria antitoxin and other horse-serum products.

Dr. Smadel added that he did not mean to suggest that the two diseases were identical, but only that there might be some general relationship between the etiological agents, just as there was between the viruses of Japanese B encephalitis and yellow fever.

Dr. Murray noted that one difference already apparent between the agent of infectious anemia of horses and that of homologous serum hepatitis was that the latter was more heat resistant. Hepatitis sera were still infective after they had been heated for four hours at 60° C. On the other hand, heating at 55° C. for 30 minutes, as required of all veterinary sera, apparently inactivates the infectious anemia agent.

Dr. Stokes suggested that the results of clinical studies of hepatitis might have some bearing on studies of the possible relationship of bacteria to the growth of the hepatitis virus. It was well known that outbreaks of diarrhea frequently preceded epidemics of hepatitis in World War II, and in many more recent studies in the United States similarly related outbreaks, sometimes due to Salmonella, had been noted. The beneficial effects of aureomycin in chronic hepatitis suggested that eradication of bacterial flora might have something to do with the course of the disease. In addition, it had been shown that germ-free animals maintained on diets deficient in the sulfhydryl-containing amino acids did not develop hepatic necrosis, as did those which were not germ-free. The necrosis was most often localized in the left lobe of the liver, which receives blood from the lower bowel where there is the greatest population of bacteria. Other instances of combined agents causing disease, such as Eperythrozoön and MVH in mouse hepatitis, supported the pos-

sibility that the agent of hepatitis might be propagated by inoculation of hens' eggs or tissue culture with bacteria or their products simultaneously with the virus itself.

It has also been shown, in the studies made by Dr. Gauld and by Dr. Neefe, that volunteers who had previously had serum hepatitis were more susceptible to subsequent infection with epidemic infectious hepatitis. The implication of this finding was that one virus, or its toxin, might make liver cells more susceptible to infection with the other virus. This possibility should be considered in work with tissue cultures.

Clinical studies had also shown that whereas gamma globulin protected against IH, even convalescent gamma globulin did not protect against SH. As Dr. Murray had indicated, this fact should be kept in mind in studies in which attempts were made to infect tissue cultures.

Dr. Enders stated that he first wished to report on tissue culture experiments in which he had employed four of the materials provided by Dr. Murray: NIH-8, L-179, L-125, and L-154. These materials were inoculated into cultures of human kidney, of human embryonic skin and muscle, and of human embryonic lung. The medium employed consisted of amniotic fluid, embryonic extract, and horse serum. The first cultures made were carried for three months and, although they showed occasional minimal degeneration, there was no definite effect except in one group. On about the 16th day it was noted, in the group of embryonic lung cultures inoculated with NIH-8, that the plasma had begun to slip badly and had disappeared rapidly from the sides of the tubes. In all of other cultures the plasma had remained intact for long periods. Accordingly, the cultures which had shown slipping of the plasma were passed again in embryonic lung tissue; slipping occurred again. A third passage had been under way for six weeks at the time of this meeting, and plasmolysis had not yet occurred. This finding excited some interest because Carrell had once reported experiments in which he infected monocytes with Rous sarcoma virus, and the cells began to digest plasma very actively, perhaps because they became tumor cells which have a tendency to do this.

In another study both stool and plasma from the five cases of infectious hepatitis mentioned earlier by Dr. Enders were inoculated into suspended cell cultures of human kidney, which were kept going for some time. Fluids from these cultures were then put in roller tube cultures of various human tissues but, again, only occasional minimal and insignificant changes were found. This work had been stopped when it was found by Dr. Murray that one of the supposedly infective plasma samples used did not cause hepatitis in human volunteers.

It was possible, too, that the hepatitis virus might not be cytopathogenic in tissue cultures in the conventional way, but might somehow change the metabolism of the cells. Valuable observations might be made from studies of the status of enzyme systems in cells to which the virus had been added.

Another approach to the study of hepatitis which Dr. Enders suggested was the application of the fluorescent antibody technique first described by Coons[1]. Coons had recently produced rabbit antibodies against human gamma globulin and then coupled fluorescein to the antibody by treatment with fluorescein isocyanate; the conjugated antibody retained its specificity. Tissue specimens which were suspected of containing virus were then treated with the labelled antibody, and it was found to become localized in the areas where virus was present. The method was practical and had worked very well in certain cases.

Willard and Coons had recently applied this method to chickenpox virus grown in roller tube tissue cultures. Labelled convalescent serum lighted up the foci in the culture where the chickenpox virus had localized; labelled acute phase serum did not. Results of great regularity were obtained with this method, using a number of specimens of acute and convalescent chickenpox serum. The method might well be applied to hepatitis, using liver slices from autopsy material and sera taken from volunteers before and after inoculation with infective material.

Dr. Snyder commented that the fact that the Coons labelled-antibody method had worked well in studies of Rubarth's canine hepatitis[2] added to its prospective value in studies of human hepatitis material.

Dr. Smadel commented that his chemist had had some difficulty in preparing adequate amounts of the materials needed for labelling antibodies with fluorescein, and asked whether Dr. Coons had the materials available in large amounts.

Dr. Enders said that the method itself was simple, requiring only one fluorescent antibody for any antigen. It was an extremely valuable technique, and the necessary materials could be made widely available.

Dr. Werner Henle recalled that he had explored a related method, but with unsatisfactory results. Gamma globulin was labelled with radioactive iodine and suspensions of influenza virus were added. When the virus was centrifuged out, it was found that the counts in the precipitate were no higher than those found in precipitates of labelled globulin solutions to which virus had not been added, so the method had been abandoned. It was probably not as sensitive as the fluorescein method.

Dr. Enders added that the Coons method could be applied not only to liver biopsy specimens, but also to tissue cultures and even cover-slip preparations of cells.

He then stated that Dr. Havens' report of the many nonspecific serological reactions occurring with hepatitis antigens made him recall the nonspecific complement-fixing reactions which occasionally occurred between normal human serum and mumps-infected egg material. Although infected egg material would fix complement, mumps antigen did not, nor did normal egg material. This suggested that at some time

---

[1] Coons and Kaplan: J. Exper. Med. *91*:1, 1950.

[2] Coffin, Coons, and Cabasso: J. Exper. Med. *98*:1, 3, July 1953.

during the course of the egg infection, abnormal antigenic materials were produced. Some individuals might normally have antibodies against these abnormal egg antigens, and these probably caused the nonspecific complement-fixing reactions and nonspecific positive skin tests observed with infected egg materials. It might be worth while if someone would systematically study these nonspecfiic substances which appear in infected materials and cause so much confusion in the serological study of virus diseases.

Dr. Enders also suggested that it might be possible to remove the inhibitory factors present in serum which prevent growth of virus in small systems like tissue cultures. Such serum might be capable of producing infections when injected into the human body, where the inhibiting factors would be diluted considerably, whereas the high concentration of inhibitory factors would present growth in tissue culture. Experiments might be tried in which proteolytic enzymes would be allowed to digest the serum before it was inoculated into tissue culture, in the hope that the virus inhibitors could be removed by enzymatic digestion.

Dr. MacCallum's observation that hepatitis might not be primarily a disease of the liver, but rather of the gastrointestinal tract, suggested that attempts should be made to establish cultures of intestinal tissues and then infect them with hepatitis virus. Considerable study of methodology would be required to maintain these tissues as organoid cultures. However, it might be that such cells are the ones in the body in which the virus propagates; this localized virus infection might later affect the liver indirectly, perhaps in a fashion analagous to the late demyelinating effects of measles.

In his opinion, the search for an etiological agent should probably be concentrated for the present on IH, since this was the disease which was most clearly due to an infectious agent.

Dr. Bang asked whether there had been any experiments which demonstrated that virus might be separated from antibody by centrifugation. That possibility had been one of the reasons for the centrifugation used in his tissue culture studies.

Dr. Enders recalled that Dr. Sabin had done such studies.

Dr. Sabin said that the ability of centrifugation to dissociate antibodies from viruses varied with the different viruses. In the case of vaccinia, mixtures of virus and antibody which did not produce disease in rabbits had been centrifuged at a speed which sedimented the virus, and almost quantitative recovery of infective virus was obtained. In subsequent experiments with influenza virus, which were conducted by Dr. Francis, it was shown that separation became more difficult the longer the influenza virus was in contact with the antibody. The vaccinia virus, however, could still be recovered from antibody mixtures even after several days of incubation.

Dr. Enders noted that he had centrifuged mixtures of pneumococci with neutralizing serum, similar to Dr. Sabin's virus mixtures, and had found that the precipitated pneumococci, despite capsular swelling, were as virulent for mice as they had been before the neutralizing serum had been added.

Dr. Sabin added that the centrifugation process had also been employed in the adaptation of dengue virus to mice; the centrifuged material infected mice more readily. Centrifugation probably achieved much the same quantitative effect as dilution, since it decreased the proximity of antibody to viruses in mixtures, but it avoided the danger of concomitant excessive dilution of the virus.

Dr. Enders asked what was known about the amount of hepatitis virus in the cells as compared with the plasma. Were washed cells infective?

Dr. Murray replied that he did not know whether this problem had been investigated.

Dr. Werner Henle said that the question had been considered before, but the necessity of using human volunteers had prevented a solution.

Dr. Sabin noted that in many cases it was quite possible that the cells from an immune host might be more infectious than mixtures of serum and cells. Dr. Bang's reported studies with Newcastle disease recalled experiments which Dr. Sabin had conducted 20 years before, in which it was found that if whole blood infected with vaccinia virus were inoculated into hyperimmune rabbits, disease did not develop, but if the centrifuged white cells were injected, the disease appeared.

Dr. Enders added that in Dr. Weller's experiments with chickenpox in tissue culture, the virus could be propagated in series almost indefinitely, with the infection of the majority of cells occurring by direct and immediate contact with other infected cells. Culture fluid free of cells was not infectious. Perhaps if cells containing the virus were added to tissue cultures, the virus might multiply more readily.

Dr. Stokes recalled that the impression had been gained from Dr. Rake's study with measles virus that the white cells were very definitely associated with the virus.

Dr. Smadel then made the plea that whenever laboratories doing tissue culture work came across "orphan viruses," (Dr. Melnick's term for viruses in search of a disease), these should be tested with paired sera from cases of infectious hepatitis. It might well be that hepatitis virus had already been propagated in tissue culture somewhere, but had not been identified by subjecting it to specific neutralization tests for IH.

Dr. MacCallum wondered at what stage in the production of gamma globulin from plasma by the cold ethanol process the hepatitis virus was destroyed. Even infected plasma so sequentially fractionated yielded

safe gamma globulin, but outright addition to infected material of the various chemicals used in the procedure did not kill the virus.

Dr. Murray indicated that the answer to this question was not known. The alcohol fractionation technique, in which some heating was employed, produced gamma globulin which did not cause hepatitis in clinical use or in experimental testing. However, gamma globulin prepared from infected plasma by the zinc precipitation technique produced hepatitis in all of five inoculated volunteers.

Dr. Stokes commented that Dr. Enders had shown that such prototypes of the hepatitis virus as tobacco mosaic and mouse encephalitis virus, when added to plasma before cold ethanol fractionation, were spread through all of the products. For reasons unknown, this spreading apparently did not occur with the serum hepatitis virus in plasma fractionated by the cold ethanol method. On the other hand, Dr. Murray's results with gamma globulin prepared by the zinc precipitation technique suggested that inordinate concentration of the virus in the gamma globulin fraction resulted from this method.

Dr. Snyder observed that it might be important in tissue culture efforts to recreate in the test tube, temporarily at least, the environment which was present in the intestinal tract, with the idea that the virus can attach to, or penetrate into, the cell only with the facilitation of the medium present in intestinal fluids. Such a medium was obviously not a good one to have surrounding the cells for a long time, but it might be useful in helping to initiate the infection.

Dr. Sabin then elaborated on Dr. Enders' comment that the hepatitis virus might well multiply in tissue culture and yet not produce any cytopathologic effect by which it could be detected. Japanese B encephalitis virus could multiply in both HeLa cells and in monkey kidney cells without producing cytopathologic changes. It might be highly worthwhile to take this system as an initial model to find out what metabolic processes of the tissue culture cells might be affected by a process of inapparent virus multiplication. The same metabolic factors might be altered by contact between the hepatitis virus and tissue culture cells. One should probably use not only the roller tube method, but also employ the liver slice technique, perhaps beginning with such simple studies as the measurement of glucose utilization in the presence of infected and noninfected human serum. Such chemical methods might well be indicators of virus multiplication in the in vitro tissue preparations.

Dr. Sabin concluded by stating that he considered this meeting unique in presenting such an encouraging display of perserverance and patience in the face of frustrating experimentation.

Dr. Tillett added that he felt the meeting had well served its intended purpose of producing a careful documentation and evaluation of past work, and that many promising routes for future study had been delineated.

On behalf of the National Research Council, Dr. Tillett then thanked the participants for their worth-while efforts.

Dr. Bayne-Jones expressed his appreciation to the participants on behalf of the Research and Development Division of the Department of the Army, and stated that many new efforts and approaches should result from the discussion.

Dr. Paul then concluded the Symposium with an expression of his gratification at the successful collaboration between the National Research Council and the Armed Forces Epidemiological Board which had made this Conference possible. He felt sure that the many new ideas brought forth for detection of the virus, such as the application of the Coons technique and the variety of new approaches in tissue culture including the use of metabolic studies of tissue cultures, had already stimulated each of the participants who had a laboratory to consider new and promising programs of study. He recalled that he had had initial misgivings about this meeting, having been haunted by a fear that it would be just another discussion prompted by desperation, but now he and others present had been greatly heartened by the discussion, and could leave the Symposium with a feeling that much progress had been made.