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Interactions between complement and cellular mediated mechanisms of monoclonal antibody therapy

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INTERACTIONS BETWEEN COMPLEMENT AND CELLULAR MEDIATED MECHANISMS OF MONOCLONAL ANTIBODY THERAPY

by

Siao-Yi Wang

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Immunology
in the Graduate College of
The University of Iowa

May 2010

Thesis Supervisor: Professor George J Weiner

ABSTRACT

Monoclonal antibodies (mAbs) have become an important part of therapy for a number of cancers. The first mAb to be approved for clinical use is rituximab, which is currently used for the treatment of various B cell malignancies. Despite its clinical value, the mechanisms in which rituximab induces tumor regression are unclear. Growing evidence suggests that multiple mechanisms involving complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) are involved. However, the direct interactions between CDC and ADCC have yet to be investigated.

My studies examine the relationship between complement fixation and the activation of NK cells by utilizing in vitro assays, a syngeneic murine lymphoma model, and clinical samples from patients. Using these systems, I demonstrate that the initiation of the complement cascade inhibits NK cell activation and ADCC induced by rituximab in vitro. I also show that depletion of complement enhances the activation of NK cells and improves the efficacy of mAb therapy in a murine model. Lastly, I demonstrate that NK cell activation correlates with decreased complement activity in patients after rituximab treatment.

The studies described in this dissertation have furthered the understanding of the mechanisms involved in antibody therapy. These results have described a novel inhibitory role for complement activity in the anti-tumor responses of mAbs. Furthermore, these findings suggest that strategies to circumvent the inhibitory effect of complement may improve how current mAbs are used and the how mAbs are designed in the future.

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Graduate College
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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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Monoclonal antibodies (mAbs) have become an important part of therapy for a number of cancers. The first mAb to be approved for clinical use is rituximab, which is currently used for the treatment of various B cell malignancies. Despite its clinical value, the mechanisms in which rituximab induces tumor regression are unclear. Growing evidence suggests that multiple mechanisms involving complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) are involved. However, the direct interactions between CDC and ADCC have yet to be investigated.

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The studies described in this dissertation have furthered the understanding of the mechanisms involved in antibody therapy. These results have described a novel inhibitory role for complement activity in the anti-tumor responses of mAbs. Furthermore, these findings suggest that strategies to circumvent the inhibitory effect of complement may improve how current mAbs are used and the how mAbs are designed in the future.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

GENERAL INTRODUCTUCTION

Development of monoclonal antibodies

Antibody therapy is a concept first introduced by Paul Ehrlich in the late nineteenth century. Through a series of studies, the "side-chain theory" was developed, which proposed that living cells produce side-chains associated with particular toxins. This theory also hypothesized that the specific side-chains are released to neutralize these toxins and that the ability to selectively target a disease-causing agent without damaging normal tissue could serve as a "magic bullet" (1). However, the use of antibody-based serotherapy to treat disease has had limited success. Sera from immunized animals are inconsistent and result in host immunogenic responses. These obstacles prevented the effective use of antibody therapy and the development of "magic bullets".

Subsequent studies revealing the structure and function of antibodies have advanced the field of antibody therapy. Produced by plasma cells, the basic structure of an antibody consists of two large heavy chains and two small light chains connected by disulfide bonds (2) (Figure 1). Each chain contains structural domains that can be classified as a constant or variable domain (3). In mammals, there are five different types of antibody heavy chains ($\alpha,\delta,\varepsilon,\gamma,\mu$), which determine the class (isotype) to which the antibody belongs (IgA, IgD, IgE, IgG, IgM) (4). In addition, there are two types of mammalian antibody light chains (λ,κ), which form the Fab (fragment, antigen binding) region of the antibody along with portions of the heavy chains (5). The variable domains of the heavy and light chains form the Fv region of the antibody, which is crucial for binding to specific targets, known as an antigen (4). The Fc (Fragment, crystallizable) region of the antibody is located at the base of the structure and is composed of two constant domains of the heavy chains. This portion of the antibody plays a role in modulating immune response through the binding of specific proteins, such as Fc receptors or complement proteins (6).

Investigations involving multiple myeloma demonstrated that malignant B-cells produce a single type of antibody. Furthermore, the development of the hybridoma technique by Kohler, Milstein, and Jerne has made the production of identical antibodies to a specific antigen possible. The fusing of a non-antibody producing myeloma cell line to healthy B-cells from immunized mice results in antigen-specific monoclonal antibodies (mAbs) (7), addressing the problems with specificity and consistency. Numerous mAbs that target various malignancies have since been developed using the hybridoma technique. However, early clinical trials have shown that murine mAbs are immunogenic and have suboptimal interactions with the human immune system.

Recent advances in molecular biology and DNA recombinant technology have allowed the DNA encoding the binding portion of murine mAbs to be combined with DNA encoding human antibodies. By replacing the murine Fc portions with human Fc, "chimeric" mAbs are produced in which the specificity towards the antigen is retained. These chimeric mAbs have a longer half-life, limited immunogenicity, and an enhanced ability to engage the human immune system (Figure 2). The development of new techniques, such as the immunization of transgenic mice expressing human immunoglobulin genes, has made it possible for mAbs to be completely humanized (8).

Use of monoclonal antibodies in cancer treatment

The first mAb to be approved for clinical use in cancer treatment was rituximab (Rituxan), a chimeric anti-CD20 antibody. It is currently used for the treatment of CD20-positive B cell malignancies as well as some autoimmune disorders. The approval of trastuzumab (Herceptin), a humanized anti-Her2/neu mAb for the treatment of breast cancers overexpressing Her2/neu, and alemtuzumab (Campath), a humanized anti-CD52 mAb for the treatment of B-cell chronic lymphocytic leukemia, soon followed. Several other mAbs have now been approved by the FDA, and many more are currently in clinical trials for the treatment of various malignancies. Some directly target tumor cells

while others employ alternative strategies, such as targeting tumor vasculature or angiogenic growth factors (9, 10).

The clinical value of mAbs in cancer treatment was demonstrated by the initial studies of rituximab, where single agent treatment of patients with relapsed low-grade non-Hodgkins lymphoma had a 48% overall response rate and limited toxicity (11). Despite this success, there is still a need to improve mAb therapies. In the rituximab study described above, only half of the patients achieved a partial or complete response and the median duration of response was only 12 months (11, 12). Subsequent studies have shown that resistance to single agent rituximab eventually develops in almost all patients. Other mAbs have not been as effective as rituximab. In the Phase III study of single agent trastuzumab, the overall response rate was only 15% (13).

Mechanisms of tumor lysis

Even though ten mAbs are currently approved for cancer treatment, the mechanisms of action responsible for their clinical efficacy, or lack thereof, are still unclear (14-16) (Table 1). Understanding the mechanisms by which mAbs mediate tumor regression is crucial to the design of more effective therapies.

Various studies, including both in vitro and animal models, suggest that transmembrane signaling, complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC), all may play a role in the direct anti-tumor effects of mAbs. MAbs of different subclasses have varied abilities to mediate CDC and ADCC. Most anti-cancer mAbs in use and under development are of the human IgG1 isotype, which is effective at both fixing complement and engaging effector cells. For rituximab, a chimeric IgG1, CDC and ADCC appear to be particularly important (17). However, different models of study suggest one or the other as the crucial mechanism. Information from correlative clinical studies is also conflicting. It remains unclear

whether the tumor regression observed in patients treated with rituximab is due to CDC, ADCC or a combination of the two.

Other mechanisms of action are also important for select mAbs. MAbs against angiogenic factors do not directly target tumor antigens, thus their anti-tumor effects do not likely involve CDC or ADCC. By targeting VEGF, bevacizumab inhibits the formation of vasculature needed to support tumor growth, thus affecting the tumor indirectly (18). Other mAbs are primarily designed to interfere with ligand-receptor signaling. Cetuximab, which binds to surface EGFR, and trastuzumab, which targets HER2, can induce tumor cell death by impacting cell signaling. However, they also have the potential to induce anti-tumor effects through CDC or ADCC.

In this chapter, I will focus on mechanisms of direct anti-tumor activity and explore the evidence supporting the role for CDC and for ADCC. I will also explore the possible interactions between the two mechanisms and the implications they have on the therapeutic activity of mAbs.

Role of complement-dependent cytotoxicity

The fundamentals of the complement cascade, including its activation by antibodies, was first described in the middle of the last century (19). Antibody-mediated CDC is initiated when C1q, the initiating component of the classical complement pathway, is fixed to the Fc portion of target-bound antibodies. Once C1q binds to the antibody Fc, C1r cleaves itself and becomes activated, which then cleaves other C1r and C1s molecules. Once C1s is cleaved, it becomes a protease that binds and cleaves C4 and C2. When C4 is cleaved into C4b, a thioester bond is exposed, which is mostly hydrolyzed by water. However, a minority of C4b becomes covalently bound to the target surface. When C2 is cleaved, the C2b fragment forms a complex with the C4b bound to the pathogen. The C4b2b complex becomes the C3 convertase of the classical pathway,

which cleaves C3 into C3b and C3a fragments. C3b is then deposited on the target surface (4).

The deposition of C3b is amplified by the alternative pathway, which involves factors B and D. The input signal for this pathway involves factor B binding to a C3b fragment already bound to the target surface. The C3b and factor B complex becomes susceptible and is cleaved by factor D into C3bBb, the C3 convertase of the alternative pathway. Because C3Bb acts like C4b2b in creating more C3b, which in turn creates more C3Bb, the alternative pathway becomes a positive feedback system and amplifies the reaction started by C4b2b. This leads to C3b saturation of the pathogen surface (4).

As the complement cascade progresses, C3b can also bind to the C3 convertase itself, forming C4b2b3b or C3b2Bb. These new complexes are called C5 convertases, which cleaves C5 into C5b and C5a. C5b initiates the formation of the membrane attack complex. C6 and C7 bind to C5b, which exposes a hydrophobic site in C7 and inserts into the pathogen lipid bilayer. C8 then binds to C7 and also inserts into the bilayer, initiating the membrane attack complex (MAC) pore formation by the C9 components (4). (Figure 3).

Because of the positive feedback in the complement cascade, regulation is needed to prevent the system from causing harm to the host. One regulatory protein, the C1 inhibitor, binds to activated C1r and C1s and causes it to dissociate from C1q, which limits the amount of cleavage of C4 and C2 (20). C4-binding protein also limits the production of classical C3 convertase by binding to C4b and displacing C2b allowing for degradation by factor I. Factor H similarly binds to C3b and allows it to be degraded by factor I (4). Host cells themselves also have surface molecules that inactivate C4b and C3b fragments they may have bound to their surfaces. Decay-accelerating factor (CD55) is expressed by host cells and binds to C3b and C4b of C3 convertase, causing dissociation (21). Membrane co-factor protein (CD46) also binds to C3b and C4b, making them susceptible to cleavage by factor I (22). CD59 helps to regulate the

complement system through inhibiting the formation of the membrane attack complex. By binding C5b678, CD59 prevents C9 from binding and polymerizing (23).

Several in vitro studies have demonstrated that rituximab is highly efficient in mediating CDC on various B cell lines as well as fresh samples (17, 24-29). In addition, small differences in the epitope specificity of anti-CD20 mAbs can have a significant impact on their ability to mediate CDC (30). A major factor responsible for this epitope sensitivity appears to be how mAb binding and cross-linking impacts the redistribution of the target antigen into lipid rafts on the cell surface. The density of such complexes within the lipid rafts and the positioning of the antigen-antibody complex effects C1q binding, which in turn impacts the complement cascade and CDC (30, 31).

As with anti-CD20 mAbs, alemtuzumab has also been shown to redistribute its target through lipid rafts (32). In addition, the level of complement mediated lysis induced by alemtuzumab correlates with the CD52 expression on the target cells (33). In contrast, trastuzumab has been shown to downregulate, rather than redistribute, its target. This may explain why in vitro studies have demonstrated only a limited ability for trastuzumab to induce CDC (34, 35). The data to date indicate a varied ability for mAbs to mediate CDC, which implies that complement-mediated lysis may play a more important role in the clinical activity of some mAbs compared to others.

Several in vivo tumor models have provided further evidence that the anti-tumor activity of rituximab is dependent, at least in part, on complement. Cragg and colleagues demonstrated that depletion of complement reduces the therapeutic activity of anti-CD20 mAbs in xenografts of human B cell lymphomas in immunodeficient mice (30). Di Gaetano and colleagues found that rituximab induces tumor regression of syngeneic EL4 cells transfected with human CD20 in immunocompetent wild type, but not in C1q-deficient mice. Furthermore, depletion of NK cells or polymorphonuclear cells did not affect the therapeutic activity of rituximab in this model (36). However, EL4 is a T cell lymphoma that has less expression of the complement activation inhibitor, CD59, than

most B cell lymphomas (37). This could explain the sensitivity of CD20 expressing-EL4 cells to rituximab-mediated CDC. Additionally, the same team of investigators transfected the murine B cell lymphoma, 38C13, with CD20 to evaluate the importance of CDC. Using this model, they found that complement was central to the therapeutic response to rituximab (38). Interestingly, when the 38C13 tumor is targeted by an anti-idiotype antibody, rather than anti-CD20, ADCC appears to be the predominant mechanism (39). To date, there have been no definitive in vivo experiments to suggest that CDC is important in the therapeutic response to trastuzumab or alemtuzumab. Taken together, these findings suggest that the target antigen, tumor system and mAb may all be important factors in determining the importance of CDC as a mechanism of action.

Clinical observations provide evidence that complement is activated during treatment with rituximab. Complement activation correlates with the infusional toxicity sometimes seen in patients with high numbers of circulating B cells (40). Studies on complement activation inhibitors show that CLL cells that are not cleared after rituximab treatment have a higher expression of surface CD59 (41, 42). Follicular lymphoma cells, which are more sensitive to rituximab clinically, are more effectively lysed by complement than are large cell lymphoma or mantle cell lymphoma cells, which tend to be more resistant to single agent rituximab (43). However, clinical studies have failed to show that expression levels of complement inhibitors predict the clinical outcome of rituximab treatment (29). Thus, overall, there is solid evidence that complement can be an important mechanism of action of mAb in the laboratory, but the clinical evidence is less clear.

Despite the conflicting evidence related to the role of complement in mAb therapy, several strategies have been devised to enhance CDC. One approach is to neutralize the complement regulatory proteins, CD46, CD55, and CD59. These glycosylphosphatidylinositol-anchored proteins are expressed on a variety of malignant B cells and may protect them from complement-mediated killing (18, 19). CD46

(membrane cofactor protein) is a cofactor for the cleavage of C3b and C4b while CD55 (decay-accelerating factor) accelerates the decay of C3 and C5 convertase. CD59 inhibits complement-mediated lysis by preventing the formation of the MAC pore. MAbs that block these proteins increase the CDC activity of rituximab in vitro (25, 28, 42, 44). In addition, siRNA against CD55 enhances the ability of rituximab to mediate CDC in vitro (45). Stabilizing activated complement components has also been considered as a way to enhance CDC killing of tumor cells. A mAb to C3b, a central factor in complement activation, can increase C3b deposition on the surface of B cell lymphomas and result in increased complement killing in vitro (46). With the recent advances in mAb engineering, the development of mAbs with enhanced CDC activity has also become a possibility. Thus, producing a mAb with a higher affinity for C1q is another strategy to increase CDC activity. The binding site of C1q on human IgG1 has been mapped and modified mAbs with higher C1q affinity have demonstrated higher complement-mediated killing in vitro (47, 48).

Role of antibody-dependent cellular cytotoxicity

MAbs can induce ADCC mediated by a variety of effector cells, which actively lyses a target cell that has been bound by a specific antibody. Classical ADCC involves the activation of NK cells by target-bound antibodies. When the Fcγ receptor (FcγRs) on an NK cell binds to the Fc portion of the antibody, the NK cell releases cytokines such as IFN-γ and cytotoxic granules. These perforin and granzyme containing granules enter the target cell and induce cell death through apoptosis (Figure 4). ADCC has also been demonstrated to be induced by monocytes and eosinophils.

The process of ADCC requires the binding of Fc portions of the target-bound mAb to FcγRs on the effector cell (49). The activating receptors, FcγRIa, FcγRIIa, FcγRIIc, and FcγRIIIa (CD64, CD32a, CD32c, and CD16 respectively), signal through an immunoreceptor tyrosine-based activation motif (ITAM). When the activating FcγRs are

crosslinked by IgG bound to its target, tyrosine phosphorylation occurs on the ITAM by Src tyrosine kinases, leading to recruitment of Syk family kinases (50, 51). Downstream signaling then activates the linker for activation of T cells (LAT), multi-molecular adapter complexes, and phosphoinositide 3-kinase (PI3K) (52-54). PI3K produces phosphatidylinositol-3,4,5-trisphosphate, which acts as a docking site for Bruton's tyrosine kinase (BTK) and phospholipase Cγ (PLCγ). PLCγ activation leads to an increased intracellular calcium level, leading to downstream signaling events (53, 55). The inhibitory receptor FcγRIIb (CD32b) contains the immunoreceptor tyrosine-based inhibitory motif (ITIM), which leads to the inhibition of signaling activation when phosphorylated (56, 57). When ITIM-containing proteins are activated, the SHIP and SHP1 phosphatases are recruited. Phosphoinositide intermediates are then hydrolyzed, which inhibits BTK and PLCγ recruitment (58-60). FcγRs are expressed by various cell populations. Effector cells of the innate immune system such as monocytes, macrophages, dendritic cells, basophils, and mast cells express both activating and inhibitory FcγRs. NK cells express only the activating receptor, FcγRIII, while B cells express only the inhibitory receptor, FcγRIIb (49)

Understanding of the interactions between FcγRs and antibody Fc fragments were made possible when the crystal structures of several FcRs were solved (49). Currently, crystal structures for FcγRIIA, FcγRIIB, FcγRIII, and FcεRI exist (61-64). These structures show that the basic arrangements of different FcRs are similar, despite varying specificity for their ligands (65). In addition, structural information on the antibody Fc portion was also necessary to understand FcγR-antibody interactions (66). It was found that the Fc fragment, consisting of the CH2 and CH3 domains, form a horse-shoe-like structure with sugar moieties attached to an asparagine at position 297 (N297) in the CH2 domain. More than 30 variations of these sugar moieties can be found on human IgG, which make antibodies highly heterogeneous (49). When the co-crystal structure of human FcγIIIA in complex with the Fc portion of human IgG1 was solved, it revealed

that an FcγR domain contacts both lower hinge regions of the Fc region and intercalates into the groove formed by the two chains of the Fc fragment (67, 68). The interaction between a single FcγR molecule and an Fc fragment is asymmetrical, suggesting the need for a second FcγR molecule binding to the Fc. This may explain why binding of a monomeric antibody does not lead to effector cell activation and why multiple Fc fragments are necessary to induce a physiological response.

Although much of the knowledge about FcγR activity of various antibody classes are from mouse models, there are differences between the mouse and human domains and patterns of expression that need to addressed when interpreting data. Based on the genomic location and sequence similarity, mouse FcγRIV seems to be analogous to human FcγRIIIA while mouse FcγIII is the most related to human FcγRIIA (49). In addition, mouse FcγRIII does not contain the ITAM in its intracellular domain that is present in the human FcγRIIA. There is also a receptor, FcγRIIIB, which is only expressed in human neutrophils and is attached to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor (49). Overall, the affinity of mouse FcγRs for IgGs is significantly higher than the human FcγRs. Even with these differences, mouse models have played a large part in determining the functions of FcgRs in vivo and much of the principles have been confirmed in humans.

Multiple studies have shown that mAbs are efficient in inducing ADCC in vitro. Preclinical studies demonstrate that rituximab can induce ADCC of human lymphoma cell lines by human peripheral blood mononuclear cells (17). Other mAbs, such as trastuzumab (69-71) and alemtuzumab (33, 72-75), also are able to mediate ADCC in vitro.

As is the case with CDC, it is unclear how much of a role ADCC plays in the clinical activity of mAbs. Clynes and colleagues provided strong in vivo evidence for the importance of ADCC through study of an immunocompetent, syngeneic melanoma mouse model. In this model, a melanoma-specific mAb was effective in wild type mice,

but not in mice lacking the common FcR γ chain (76). In addition, the anti-tumor activity of rituximab and trastuzumab against human xenografts were reduced in FcR γ chain-deficient mice (77). The anti-tumor effect of mAb therapy was enhanced in mice lacking the inhibitory receptor, CD32b. In these models, CD64 and CD16 were necessary for mAbs to be effective. Similar studies using an adult T-lymphoma model demonstrated that FcγRs were also necessary for alemtuzumab to induce tumor regression (78).

The most convincing evidence that ADCC is mechanistically involved in clinical response to mAb therapy comes from a series of correlative studies associating polymorphisms on CD16 with clinical response to rituximab. CD16 from patients homozygous for valine at amino acid 158 (VV) has a higher affinity for IgG1 than carriers for phenylalanine (VF or FF) (79). Patients with untreated follicular lymphoma that are genotyped as VV have a better clinical response to rituximab than patients that are VF or FF (80, 81). The same polymorphism is also predictive of rituximab response in patients with Waldenstrom's macroglobulinemia (82). Follicular lymphoma patients homozygous for histidine at amino acid position 131 (HH) on CD32a, also have an improved response to rituximab (80). These data demonstrate the importance of Fc-FcγR interactions in the anti-tumor effects of rituximab and suggests that ADCC is a major mechanism of action.

In contrast, this polymorphism in CD16 did not predict clinical response to rituximab or alemtuzumab in chronic lymphocytic leukemia (CLL) patients (83, 84). However, these studies were relatively small and CLL is generally less responsive to rituximab than is follicular lymphoma. Even though there is a lack of clinical evidence, indirect data suggest that ADCC may play a role in CLL response to anti-CD52 mAbs. The levels of alemtuzumab detected in patients are less than the concentration needed to activate complement (73, 85). In addition, the anti-CD52-mediated depletion of lymphocytes in blood and bone marrow in CLL patients occurs in isotypes that induce ADCC without complement consumption (86). There is clearly much we have to learn

about the role ADCC plays in mAb response of CLL to both anti-CD20 and anti-CD52 mAbs.

Currently, there are no data linking polymorphisms of FcγRs to patient response to trastuzumab. However, the presence of immune cells capable of mediating ADCC within solid tumor masses has been shown to correlate with response. Tumor biopsies of patients who reached partial or complete remission with trastuzumab show higher in situ leukocyte infiltration. These cells have a greater ability to mediate in vitro ADCC in responders than in non-responders (87). Breast cancer patients treated with trastuzumab and docetaxel had increased numbers of tumor-associated NK cells and increased lymphocyte expression of granzyme B, providing additional, if indirect, support for the hypothesis that trastuzumab works at least partially through a cellular dependent mechanism (88).

As with CDC, the lack of irrefutable evidence that ADCC plays a role in the clinical response of mAb has not prevented investigators from exploring ways to enhance ADCC as an approach to improve the clinical efficacy of mAbs. One such approach is to increase the affinity of the Fc portion of mAbs for FcγRs by modifying the amino acid sequence of the IgG constant region. The binding site for FcγRs on human IgG1 has been defined and mAbs have been designed to have enhanced binding affinity to FcγRs (89, 90). These mAbs mediate ADCC in vitro more effectively than wild type mAbs (60, 61). Furthermore, anti-CD20 mAbs with increased binding to FcγRs are more effective than rituximab in inducing tumor regression in a murine B cell lymphoma model (91). Another strategy to improve ADCC involves the glycosylation state of the mAb. In order for the Fc region of IgG to interact with FcγRs, Asn297 must be glycosylated to maintain the tertiary structure of their CH2 domains (92, 93). Conditions that affect the antibody glycoforms influence its ability to mediate ADCC (94). Increasing the bisected complex oligosaccharides on the Fc region of mAbs and removing fucose on the IgG N-linked oligosaccharide chain has been shown to enhance its ADCC activity (95, 96).

Nonfucosylated versions of trastuzumab also demonstrate augmented ADCC activity against breast cancer cell lines (97). A number of these modified mAbs are in early phase clinical testing.

Other approaches to enhancing ADCC involve the use of additional substances to activate the effector cells responsible for mediating ADCC. A variety of cytokines and other immunostimulatory agents have been tested and shown in preclinical models to enhance ADCC and improve the efficacy of mAb therapy. These agents target various effector cell populations (NK cells, monocytes, granulocytes) and include IL-2, IL-12, IFN-α, GM-CSF, G-CSF and immunostimulatory CpG ODN (98-103). Early phase clinical trials demonstrate combinations of mAb and these agents can be administered safely. However, it remains unclear whether clinical efficacy of mAb can be improved by the addition of agents that activate immune effectors.

Requirements for NK cell mediated ADCC

NK cells are a lymphocyte population derived from the bone marrow. As a component of the innate immune system, NK cells protect the host by eliminating pathogens without undergoing the somatic recombination of a T cell receptor or an immunoglobulin. Having the ability to destroy infected cells and being a major source of cytokines such as IFN-g and TNF-a, NK cells have a diverse role in host defense (104).

Initially identified by their ability to kill tumor cell targets without priming, NK cells also participate in tumor immune surveillance (105-108). Several investigations have demonstrated that NK cells kill tumor cell targets with low levels of major immunohistocampatibility complex (MHC) class I complexes (109-111). Further studies linking MHC to rejection of hematopoietic cell grafts led to the “missing self” hypothesis, which states that the cells lacking the MHC expressed by the NK cells are susceptible to NK-mediated attack. The current view of NK cell activation involves a balance between signals from various activating and inhibitory receptors (83-86). The

killer cell immunoglobulin-like receptors (KIRs) play a role in the "missing self" hypothesis through inhibiting the cytotoxicity of cells expressing the appropriate MHC molecules (75, 87-89).

Through discrete steps, activation of NK cells occurs when signals from stimulatory receptors outweigh those of inhibitory receptors. Once activating receptors contact target cells expressing appropriate surface ligands, NK cells undergo adhesion, granule polarization, degranulation, and cytokine production (108). CD16 is an ITAM-associated activation receptor, which recognizes and binds to the Fc portion of human IgG. The presence of CD16 on 90 percent of NK cells makes the population a strong mediator of ADCC against antibody-coated targets (108). Interaction between CD16 and human Fc has been shown to be sufficient to induce NK cell signaling, as measured by receptor down-modulation (83, 90, 91). CD16 engagement alone has been shown to induce degranulation. However, additional receptor-ligand interactions are necessary for granule polarization and ADCC to occur (83, 91).

The formation of an immune synapse through adhesion is required for effector function of NK cells. Interactions such as the binding of LFA-1 on NK cells to intercellular adhesion molecules (ICAMs) expressed on targets have been shown to induce adhesion and granule polarization. However, degranulation and cytotoxicity of the target cell does not occur without the additional engagement of CD16 (108, 112, 113). Effective NK cell-mediated ADCC seems to require separate signals for the adhesion/polarization and degranulation necessary for target cell killing. In the absence of signaling through LFA-1, CD16 signaling is also enhanced through 2B4, NKG2D, DNAM-1, or CD2 stimulation (108, 114). This suggests that in addition to LFA-1 and ICAM, other receptor-ligand interactions may act synergistically with CD16 to induce ADCC.

Factors that influence the mechanisms of cytotoxicity

As more investigations are being done on the mechanisms of mAb therapy, it is becoming clear that the relative roles of complement and cellular-mediated cytotoxicity vary with each mAb. Although it is not fully understood what determines the importance of each mechanism, it seems to be dependent on several variables.

One factor that impacts the mechanism of action of a given mAb is the target antigen. Functions of target molecules range from growth factor receptors (115-117) to regulators of cell cycle and proliferation (118, 119). Some target antigens, such as CD20, have an unclear function. The binding of a mAb to its target results in different outcomes. Some mAbs interupt signaling transduction (120), while others induce an apoptotic pathway (121). Most mAbs target molecules on the surface of the malignant cell, while others are designed to interfere with factors that are necessary for the growth of the malignant cells (122). Among the different outcomes of antigen binding, the induction of an immunologic response, namely CDC and ADCC, has been shown to be important by the studies reviewed above. Properties of the target antigen, such as translocation into lipid rafts and redistribution, clearly play a role in the ability of mAbs to induce lysis through complement mediated mechanisms (30, 31).

Additional properties of the tumor cell may impact the importance a given mechanism is to the anti-tumor activity of mAbs. Multiple molecules contribute to the interaction between immune effector cells and target cells. Interactions between ICAMs have been shown to facilitate ADCC (123-126). The expression of adhesion molecules on the target cell in the immunologic synapse could well impact on effector-target interactions and the ability of mAbs to mediate ADCC. In addition to adhesion molecules, the expression of complement regulatory proteins on the tumor cell also impacts on the mechanisms of mAbs. Resistance to CDC of various lymphoma cell lines has been shown to correlate with the expression of CD55 and CD59 (17). Although clinical data have failed to demonstrate a correlation between the expression of

complement regulators and the clinical outcome of patients treated with rituximab (20), CD55 and CD59 could be inhibiting mAbs from inducing CDC and shifting the importance towards cellular-mediated mechanisms.

Another determinant of a mAb's mechanism of action could be the site of the malignant cells. Epratuzumab and rituximab both target B cell malignancies but utilize different mechanisms to induce tumor lysis in vitro (127). Clinically, rituximab appears to be more effective in CLL patients with extensive nodal involvement, while alemtuzumab is more effective in those with extensive marrow involvement and large numbers of circulating cells (128). The studies by Clynes and colleagues outlined above, which demonstrated the importance of FcγRs for the activity of mAbs, utilized a subcutaneous model as well as a model of solid tumor pulmonary metastasis (77). In contrast, the studies done by Di Gaetano and colleagues indicating the importance of complement were done mostly using intravenous injections of lymphoid tumors (36). We know that mAbs distribute to the extracellular compartment, but remarkably little is known about the role of complement in extracellular fluid. CDC might be a more important mechanism in malignancies where the tumor cells are in the intravascular compartment while ADCC may be more important for targeting solid tumors that are less vascularized. The ability of effector cells to reach a tumor site may also influence the efficacy of ADCC within solid tumor masses. Data correlating immune cell infiltration in solid tumors with response to mAb therapy is consistent with this hypothesis (129).

Innate properties of the mAb may also determine its mechanism of action. Cragg and Glennie demonstrated that two anti-CD20 mAbs of the same isotype have different abilities to induce complement activation. B1 and IF5 both target CD20 and are both murine IgG2a mAbs. However, IF5 was more effective than B1 at redistributing CD20 through membrane lipid rafts, binding C1q, and inducing CDC (30). Furthermore, the anti-tumor activity of IF5 was found to be dependent on complement activation while B1 seems to function through signaling apoptosis. Whether a mAb will translocate its target

antigen into lipid rafts is believed to be determined by the level of cross-linking in the plasma membrane (30). Cragg and Glennie have subsequently characterized anti-CD20 mAbs based on their ability to redistribute CD20 on lipid rafts. Type I antibodies stabilize CD20 on lipid rafts and are potent inducers of CDC. The majority of CD20 antibodies are of type I, including rituximab. On the other hand, Type II antibodies do not stabilize CD20 in lipid rafts and demonstrate less binding to C1q. This results in lower levels of CDC but exhibit higher levels of direct cell death (30, 130, 131).

Finally, the mechanism of cytotoxicity for a given mAb might also vary depending on the immunological makeup of the host. The immune system has developed with checkpoints in place to prevent overstimulation and problems such as autoimmunity. Administering mAbs that target tumor antigens is one way to bypass these checkpoints and induce a focused immune response. However, properties of the host immune system may still impact on the mechanism in which mAbs act. As outlined above, polymorphisms in FcγRIIIa can affect the ability of rituximab to activate NK cells and mediate ADCC (80-82) while the inhibitory receptor, FcγRIIb, impacts the regulation of ADCC (77). Polymorphisms within the complement system may also be affecting the ability of mAbs to induce CDC (132). The evaluation of both inhibitory and activating mechanisms will be important to maximize the efficacy of mAbs.

Interactions between mechanisms

CDC and ADCC are not totally independent mechanisms. There are several interactions between complement activation and cellular cytotoxicity that may mediate cooperation between the two. In fact, ADCC and CDC induced by rituximab have been shown to be additive in a number of models (133). Studies done by Van Meerten and colleagues found that ADCC and CDC are able to act simultaneously, resulting in greater killing than either mechanism alone. ADCC resistant cells were sensitive to CDC and

CDC resistant cells were sensitive to ADCC, providing further evidence that these mechanisms may both be important (134).

As the complement cascade is activated, the cleaved fragments of C3 and C5 become a point of interaction between complement and cellular-mediated mechanisms. C3a and C5a have potent chemotactic and inflammatory properties that can enhance the recruitment and activation of effector cells. C5a has been shown to induce upregulation of CD16 (an activating receptor) and suppress CD32 (an inhibitory receptor), resulting in an overall effect of enhancing the activation of macrophages (135). In physiological conditions, complement activation could be facilitating ADCC by recruiting effector cells and priming them to interact with mAb-coated targets.

Other points of interactions between complement and cellular cytotoxicity involve the complement receptors present on effector cells. Activation of the complement cascade results in target cell opsonization by C3b, which is recognized by complement receptors on macrophages and NK cells. Binding of C3b to complement receptors leads to phagocytosis or cell lysis through a process known as complement dependent cellular cytotoxicity (CDCC). Since studies have shown that rituximab is able to moderately induce CDCC at best (15, 16), it is unlikely that it plays a significant role on its own. However, it has yet to be ruled out whether CDCC works to enhance ADCC.

In addition to binding to C3b, complement receptors also act as a signaling partner for various FcγRs. The complement receptor, Mac-1 (CD11b/CD18), plays an important role in FcγR mediated cytotoxicity toward tumor cells (136, 137) and was found to be vital in mediating the anti-tumor response of mAbs in a mouse model known to be dependent on ADCC (138). These studies suggest that Mac-1 functions as both a complement receptor and as an accessory molecule to FcγR in mediating ADCC. Thus, complement and cellular-mediated cytotoxicity have overlapping effects, which may contribute to the overall efficacy of mAbs.

Alternatively, some studies suggest complement and cellular cytotoxicity may be antagonistic mechanisms of action. Lefebvre and colleagues found that serum inhibited rituximab-mediated killing of CLL cells by macrophages (139). Taylor and colleagues found that CD20 is cleared from the surface of CLL cells following treatment with rituximab, and have proposed that many of the target cells are not eliminated by complement, but that instead, the antigen-mAb-complement complexes on the surface of the malignant cells are "shaved" off by phagocytic cells. If this is, indeed, the case, complement fixation could actually render cells resistant to mAb therapy (140).

Additional Fcγ receptor mediated mechanisms

In addition to ADCC, FcγRs mediate other important functions of the immune system. Phagocytosis of IgG-coated microorganisms or soluble proteins by macrophages is crucial to the reticuloendothelial system and can be initiated through the binding of FcγRs (49). Once the activating FcγRs are engaged, signal transduction induces the recruitment of proteins from the cytoplasm to form a complex with the receptor. These complexes activate proteins that regulate actin, leading to the reorganization of the actin cytoskeleton and membrane remodeling (141, 142). The progressive interaction between FcγRs and IgGs proceeds in a "zipper-like" manner until internalization is completed, forming a phagosome. Fusion of the phagosome with lysosomes results in rapid degradation of the engulfed material (141).

Dendritic cells (DCs) also express FcγRs and internalize IgG-coated particles through phagocytosis. In addition, peptides derived from the phagosomes are presented by DCs in MHC class I and MHC class II molecules (49). This FcγR-mediated mechanism has been shown to stimulate a stronger immune response than fluid phase antigen internalization lacking FcγR interactions (49, 143). Engagement of FcγRs leads to maturation of DCs, upregulation of costimulatory molecules, and the induction of an adaptive cellular immune response.

Even though activating FcγRs induce the pro-inflammatory responses required to clear infections or malignant cells, destruction of healthy tissues through autoimmunity may occur without proper regulation. Expression of the inhibitory FcγRIIB on leukocytes except for NK and T cells suggests its importance in controlling the immune system. FcγRIIB signaling on B cells without B cell receptor (BCR) stimulation has been shown to induce apoptosis, preventing the development of antibody-producing plasma cells (59, 60). It is believed that this mechanism acts as a checkpoint for humoral immunity. As the most potent antigen-presenting cell population, DCs are also regulated through FcγRIIB signaling. Inhibition of FcγRIIB interactions on DCs have been shown to cause spontaneous maturation under non-inflammatory conditions (144, 145). This suggests that expression of FcγRIIB on DCs plays a major role in preventing unwanted DC activation and unnecessary immune responses. In addition, the pro-inflammatory responses of mast cells, granulocytes, and macrophages are inhibited by FcγRIIB (49). Thus, FcγRIIB is involved in maintaining the appropriate response of both the innate and adaptive immune systems.

Rationale for project

As described above, complement mediated and cellular mediated mechanisms of cytotoxicity are not completely independent. In physiologic conditions, it is likely that complement proteins and effector cell populations are both present at tumor locations. However, the direct effect of complement activation on ADCC has yet to be clearly established. There have been a limited number of studies evaluating the interactions between CDC and ADCC and the conclusions have been conflicting. For the chimeric CD20 mAb, rituximab, there is strong clinical evidence suggesting that CD16 dependent cellular-mediated cytotoxicity is an important mechanism of action. Whether complement activity facilitates or inhibits ADCC may directly impact the efficacy of rituximab in cancer treatment.

The expression of CD16 on the vast majority of NK cells makes them important mediators of ADCC. NK cells likely play a central role in the cytotoxic activity of mAbs due to their ability to induce cytotoxicity of antibody-coated targets without priming. Numerous in vitro studies have demonstrated that NK cells are efficient in mediating ADCC of target cells coated with rituximab (outlined in this chapter). This dissertation focuses on the effect of the complement cascade on NK cell activation and function induced by rituximab. In Chapter II of this dissertation, a novel in vitro assay is used to investigate the effect of complement on NK cell activation and ADCC induced by rituximab. The effects of depleting activity from various sources of complement on NK cell activation and from an in vivo mAb therapy model dependent on ADCC are explored in Chapter III. Finally, Chapter IV of this dissertation examines the correlations between serum complement activity and NK cell activation in patients treated with rituximab.

Figure 1. Schematic depiction of basic antibody structure

Antibody structure consists of two heavy chains and two light chains. The Fab region consists of one variable and one constant domain from each heavy and light chain. The Fv region is crucial to the binding of antigens. The Fc region consists of constant domains from each heavy chain. Dark grey areas depict light chain domains. Light grey areas depict heavy chain domains. Dotted circle depicts Fv region.

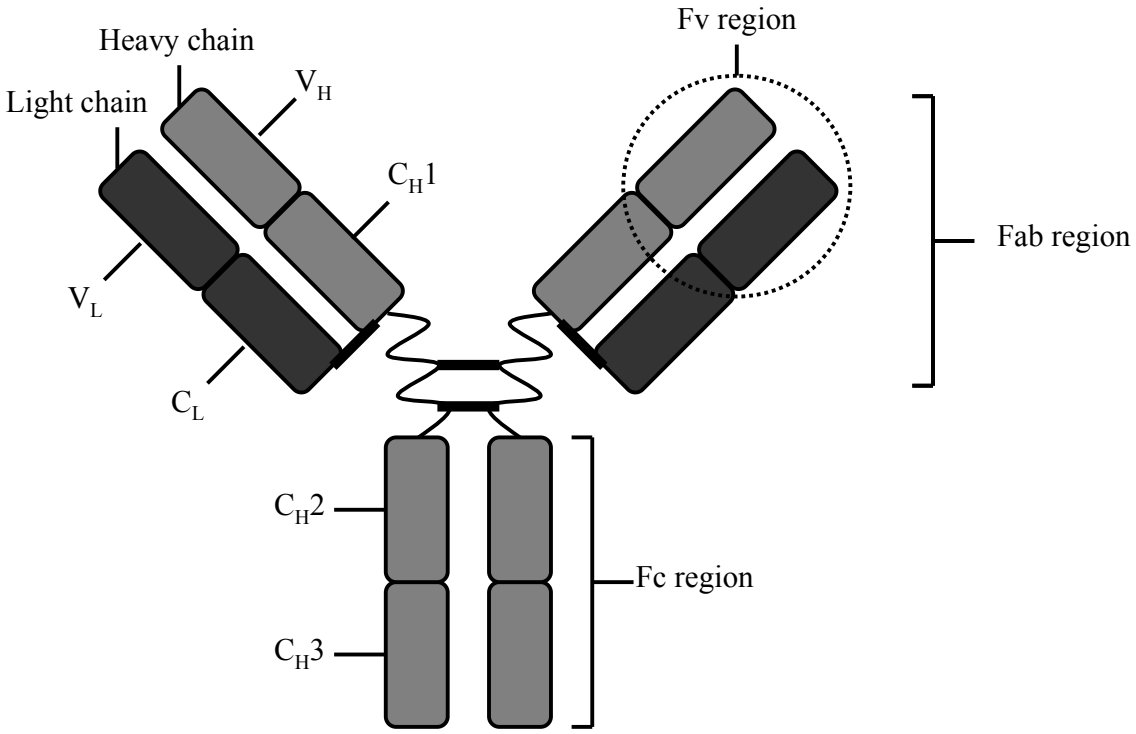

Heavy chain
Light chain
V_H
C_H1
V_L
C_L
Fv region
Fab region
C_H2
C_H3
Fc region

Figure 2. Schematic depiction of chimeric antibody structure

Antigen binding variable regions of a murine monoclonal antibody are joined to human constant regions. Dark grey areas depict murine regions. Light grey areas depict human regions.

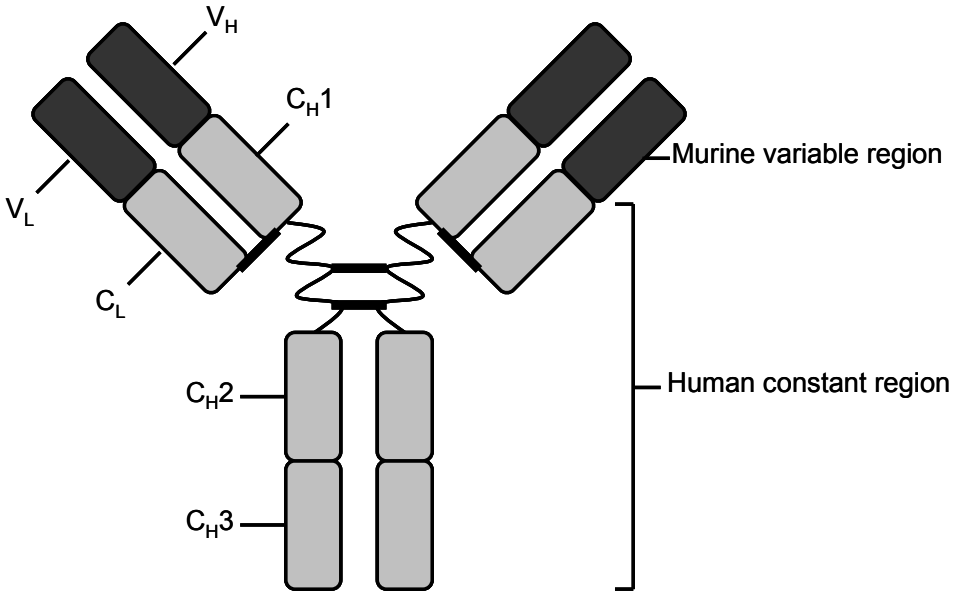

V_H
C_H1
V_L
C_L
Murine variable region
C_H2
C_H3
Human constant region

Table 1. FDA approved monoclonal antibodies for cancer treatment

Mab name	Trade name	Target antigen	Used to treat	FDA approved
rituximab	Rituxan	CD20	non-Hodgkin lymphoma	1997
trastuzumab	Herceptin	HER2/neu	breast cancer	1998
gemtuzumab	Mylotarg	CD33	acute myelogenous leukemia	2000
alemtuzumab	Campath	CD52	chronic lymphocytic leukemia	2001
ibritumomab	Zevalin	CD20	non-Hodgkin lymphoma	2002
tositumomab	Bexxar	CD20	non-Hodgkin lymphoma	2003
cetuximab	Erbitux	EGFR	colorectal cancer head and neck cancer	2004 2006
bevacizumab	Avastin	VEGF	colorectal cancer non-small cell lung cancer breast cancer glioblastoma kidney cancer	2004 2006 2008 2009 2009
panitumumab	Vectibix	EGFR	colorectal cancer	2006
ofatumumab	Arzerra	CD20	chronic lymphocytic leukemia	2009

Figure 3. Schematic depiction of complement-dependent cytotoxicity

C1q complex binds to IgG molecules, which leads to the activation of the complement cascade. The generated C3 convertase deposits large amounts of C3b molecules on the target surface. C3b binds to C3 convertase, leading to the formation of C5 convertase. The generated C5b triggers the assembly and insertion of the membrane-attack complex, which disrupts the cell membrane.

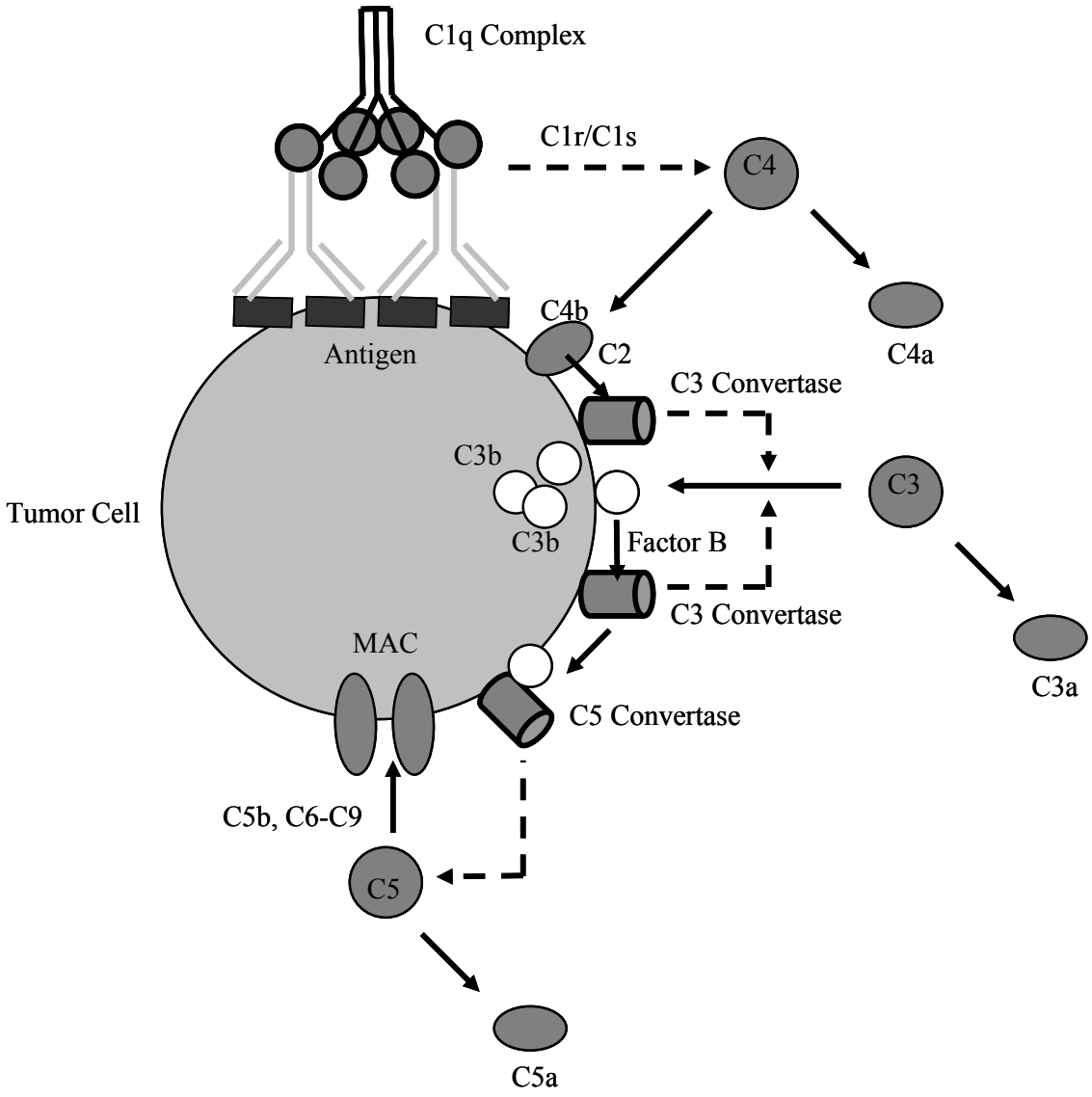
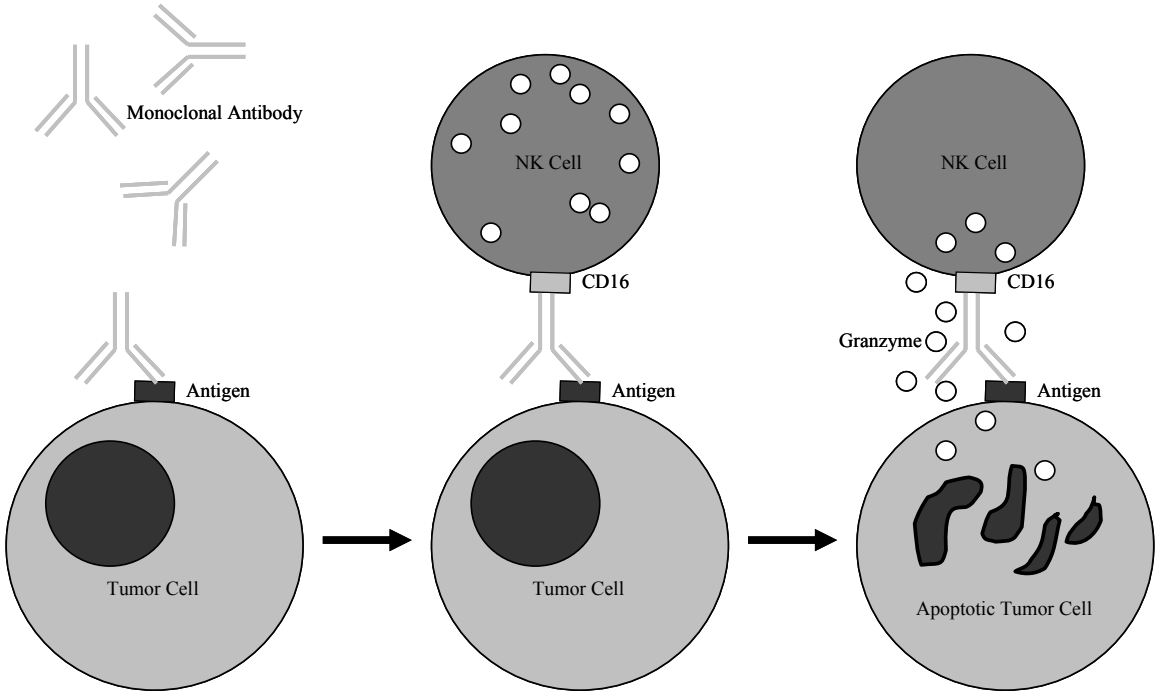

C1q Complex
C1r/C1s
C4
C4b
C2
C4a
Antigen
C3 Convertase
C3b
C3b
C3
Tumor Cell
Factor B
C3 Convertase
C3a
MAC
C5 Convertase
C5b, C6-C9
C5
C5a

Figure 4. Schematic depiction of antibody-dependent cellular cytotoxicity

NK cells have FcγRIII receptors (CD16) on their surface, which recognize the Fc portion of IgG. Binding of CD16 to IgG signals the NK cell to become activated and release cytotoxic granules. The target cell dies by apoptosis.


Monoclonal Antibody
Antigen
Tumor Cell
NK Cell
CD16
Antigen
Tumor Cell
NK Cell
CD16
Granzyme
Antigen
Apoptotic Tumor Cell

CHAPTER II

NK CELL ACTIVATION AND ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY IS INHIBITED BY COMPLEMENT ACTIVITY

Abstract

Antibody dependent cellular cytotoxicity (ADCC) and complement fixation both appear to play a role in mediating anti-tumor effects of monoclonal antibodies (mAbs), including rituximab. I evaluated the relationship between rituximab-induced complement fixation, NK cell activation, and NK cell mediated ADCC. Down-modulation of NK cell CD16 and NK cell activation induced by rituximab-coated target cells was blocked by human serum but not heat-inactivated serum. This inhibition was also observed in the absence of viable target cells. C1q and C3 in the serum were required for these inhibitory effects, while C5 was not. An antibody that stabilizes C3b on the target cell surface enhanced the inhibition of NK cell activation induced by rituximab-coated target cells. Binding of NK cells to rituximab-coated plates through CD16 was inhibited by the fixation of complement. C5-depleted serum failed to induce complement-mediated lysis but blocked NK cell-mediated ADCC. These data suggest that C3b deposition induced by rituximab-coated target cells inhibits the interaction between the rituximab Fc and NK cell CD16, thereby limiting the ability of rituximab-coated target cells to induce NK activation and ADCC.

Introduction

Monoclonal antibodies (mAbs) are now a mainstay of therapy for a number of cancers. Rituximab was the first chimeric mAb to be approved for clinical use and remains the most extensively utilized mAb in cancer therapy. Rituximab binding of CD20 has been shown to signal apoptosis in a subset of lymphoma cell lines (121). However, there is little evidence that signaling plays an important role in clinical

responses to rituximab. Growing evidence suggests multiple interacting mechanisms including complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) play a role in the anti-tumor response to rituximab and other mAbs.

Evidence is conflicting related to the role CDC plays in mediating the anti-tumor effects of rituximab. Van Meerten et al utilized target cells that express varying amounts of CD20 on their surface, and concluded that rituximab-mediated CDC depends on CD20 expression level and acts complementary to ADCC (134). Target cell expression of the complement inhibitory proteins CD55 and CD59 correlates with the ability of rituximab to induce CDC in vitro (26), and CDC is enhanced when these proteins are blocked(25). However, no correlation was found between CD55 / CD59 expression by lymphoma cells and clinical response to therapy (29). In mouse models utilizing murine lymphomas expressing human CD20, Golay et al found complement plays a key role in mediating rituximab's anti-tumor effects (27, 29). Cragg and Glennie reached similar conclusions from studies of human B cell lines in severe combined immunodeficiency (SCID) mice (146). Clinically, depletion of complement and evidence for complement fixation on target cells can be seen following rituximab therapy (140, 147). Takami et al recently described a case where supplementation of rituximab with complement by infusion of serum in the cerebrospinal fluid promoted anti-tumor activity in the central nervous system (148), suggesting complement may mediate the anti-tumor activity of rituximab in the absence of cellular immune effectors. In addition, a case was reported by Klepfish et al in which the use of fresh frozen plasma as a source of complement induced a response to rituximab in a patient previously refractory to treatment (149, 150). Nevertheless, there is no definitive evidence that complement activation correlates with or is required for clinical responses.

ADCC is another mechanism that is likely to play a central role in the response to clinical mAb therapy. Clynes et al demonstrated that Fcγ-receptor knock-out mice have a limited anti-tumor response to mAb in several tumor models (77). Most convincingly,

patients homozygous for V158 (VV) polymorphism on CD16 have higher clinical response rates to rituximab than carriers for F158 (VF or FF) (51-53). These results suggest that ADCC is a major mechanism and that CD16 plays a key role in the anti-tumor effect of rituximab.

Traditional cytotoxicity assays allow for evaluation of anti-tumor activity, but fail to differentiate the mechanisms by which target cells are lysed. Although cytotoxicity assays are the gold standard for measuring mAb induced cell lysis, chromium release can be the result of either CDC or ADCC. A co-culture assay that allows for precise measurement of mAb-induced NK cell activation was previously reported (151). In this system, peripheral blood mononuclear cells (PBMCs) and target cells are co-cultured with the mAb. Response of NK cells to mAb-coated targets is determined by phenotypic evaluation of NK cells. NK cell response is quantified by CD16 down-modulation, which follows interaction between CD16 and IgG (108, 112), and upregulation of CD69 and CD54, which serve as phenotypic markers of NK activation (151). This model was previously used to evaluate mAb with varying affinity for CD16, and found that NK activation is a reasonable surrogate for ADCC (91). In this chapter, I use these assays to evaluate the relationship between complement fixation and the ability of mAb-coated target cells to induce NKcell activation. Normal human serum was used as a source of complement. Heat-inactivation was used to neutralize complement activity, as complement proteins have been shown to be extremely heat-labile. Specifically, when the C3 component is heated above 50°C, a peptide bond in the chain is cleaved to two α chain fragments with molecular weights of 75 and 44 kDa. The original disulfide linkages connecting the two α chain fragments and β chain fragment is dissociated upon heat inactivation (152).

As outlined here, I found that complement fixation impedes NK activation induced by mAb-coated target cells through inhibiting the binding of CD16 to the mAb, leading to the inhibition of ADCC. These results indicate the relationship between

complement fixation and the clinical efficacy of mAb may be more complex than previously assumed.

Materials and Methods

Antibodies and serum

Rituximab (Biogen-Idec, Cambridge, MA; Genentech, South San Francisco, CA) was purchased commercially. The 3E7 mAb specific for C3b/iC3b was a gift from Dr. Ron Taylor (University of Virginia, Charlottesville, VA) (46). The anti-CD11b (Bear1) mAb was obtained from Biodesign (Saco, ME), the anti-CD18 mAb was obtained from BD Pharmigen (San Diego, CA). C1q-, C3-, and C5-depleted serum was obtained commercially from Complement Technology (Tyler, TX).

Samples from human subjects

Peripheral blood mononuclear cells (PBMCs) were obtained from normal volunteers after obtaining informed consent (91, 151). The studies were approved by the University of Iowa IRB. Mononuclear cells were isolated and red blood cells were removed by resuspending cells in 5 mL red cell lysis buffer according to standard procedures. Autologus normal human serum (serum) was used in select experiments at the indicated concentrations. In select experiments, an aliquot of autologous serum was heated to 57°C for 30 minutes to produce heat inactivated serum. Cryopreserved single cell suspensions of primary follicular lymphoma cells (FL) were thawed using standard procedures.

NK cell activation

Co-cultures were performed using fresh PBMCs as a source of NK cells(91, 151). Target cells included the standard human B cell lymphoma cell lines, Raji and Daudi, or FL cells. Media consisted of RPMI supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μM 2-

mercaptoethanol. PBMCs and B cells were mixed at a 1:1 effector:target ratio at a final concentration of 1 X 10^6 PBMCs cells and 1 X 10^6 B cells/mL and cultured for 20 hours at 37°C. Various concentrations of rituximab, autologous serum (from PBMC donors), heat inactivated serum, or commercially purchased C1q-depleted serum, C3-depleted serum, or C5-depleted serum were added as indicated. C5-depleted serum induced a low degree of dose dependent change in NK cell phenotype in the absence of rituximab. Therefore, for samples containing C5-depleted serum, the phenotype seen in the presence of C5-depleted serum without rituximab was used as the background level when calculating phenotypic change induced by rituximab.

NK cell phenotypic analysis

Immunofluorescent staining was performed on co-cultured cells (91, 151). Cells were washed and stained with directly conjugated commercial antibodies including anti-human CD56 AlexaFluor 647, CD54 PE, (BD Pharmingen, San Diego, CA), CD16 FITC (Serotec, Raleigh, NC) and CD3 PE-Cy7 (Caltag Laboratories, Burlingame, CA) per the manufacturer's protocol for 15 minutes on ice. Cells were washed twice, fixed in 2% formaldehyde solution, and stored at 4°C for flow cytometry within 24 hours. Four-color flow cytometry analysis was performed on the LSR II (BD Immunocytometry Systems, San Jose, CA). Data were analyzed using FlowJo software (TreeStar, Ashland, OR). CD16 and CD54 expression of NK cells was determined by gating on CD3-, CD56+ lymphocytes. CD16 expression was reported as median fluorescence. In prior studies, we found that NK cell expression of CD54 was bimodal with activated NK cells expressing bright CD54 (91, 151). Therefore, NK cell activation is reported as the percent of NK cells that were CD54 bright.

Microtiter plate adhesion assay

A microplate adhesion assay, based on a similar assay used to measure the function of bifunctional antibodies (153), was developed to evaluate the avidity of NK

cells for IgG. U-bottomed 96-well EIA/RIA microtiter plates (Corning, Corning, NY) were coated with various concentrations of rituximab overnight. Nonspecific binding was blocked by the addition of 10% heat-inactivated fetal calf serum. Media, 50% human serum, or heat-inactivated human serum was added for 30 minutes at 37°C. After washing, 5 X 10^5 purified human NK cells were added for 30 minutes at room temperature. Plates were then centrifuged for 5 minutes at 500g. Cell pellets formed when cells were not bound to rituximab-coated wells. When rituximab was present, NK cells bound to the walls of the well and no pellet was noted. The presence or absence of cell pellets was determined by evaluating single wavelength absorbance at 405 nm. Lower absorbance is seen when cells bind to the walls. Higher absorbance is seen when cell pellets form indicating decreased binding.

Cytotoxicity assay

A total of 3 x 10^6 Raji cells were labeled with 200 μCi of $Na_2{}^{51}CrO_4$ at 37°C for 1.5 hours. NK cells from normal human donors were purified from PBMCs using the NK cell isolation kit II (Miltenyi Biotec) per manufacturer's instructions. ^{51}Cr-labeled target cells were washed and dispensed into 96-well flat-bottomed plates (1 X 10^4 cells/well). Serial dilutions of effector cells (E/T ratio of 50:1 to 1:1), rituximab (5μg/ml) and C5-depleted serum or heat-inactivated C5-depleted serum (20% final concentration) were added as indicated in triplicate. After 4 hours of incubation at 37°C, ^{51}Cr activity in the supernatant was measured. The percentage of specific cytolysis was calculated from the counts of samples according to the formula: % specific lysis = 100 X (E - S)/(M - S). E represents the experimental release, S is the spontaneous release, and M is the maximum release.

Results

Normal human serum inhibits NK cell activation

It was previously demonstrated that the addition of rituximab to a mixture of target B cells and PBMCs from normal donors in a co-culture assay induces down-modulation of NK cell CD16 and upregulation of CD54 in a dose dependent manner (151). In this chapter, I evaluated the impact of serum on this effect. Serum blocked down-modulation of CD16 induced by rituximab-coated target cells at rituximab concentrations greater than 0.04μg/ml. This inhibition was observed in cultures with Raji and Daudi cell lines and FL (Figure 5A).

In the absence of serum, CD54 upregulation was noted beginning at a rituximab concentration of 0.002μg/ml, reached a plateau at a rituximab concentration of approximately 0.2μg/ml, and remained stable up 5μg/ml, the highest concentration of rituximab tested. In the presence of serum, CD54 upregulation was seen at the lower rituximab concentrations but peaked at 0.04μg/ml. CD54 upregulation decreased at rituximab concentrations greater that 0.04μg/ml when serum was present (Figure 5B). Similar results were seen when Raji, Daudi, or FL cells were used (Figure 5B); when CD69 and IFNγ were used as markers of NK activation; when purified NK cells were used in the assay (data not shown).

Inhibitory effects of normal human serum are due to complement activity

To investigate whether the inhibitory effects of serum on NK cell activation were dose-dependent, the effects of various concentrations of normal serum were assessed. The effect of serum was concentration dependent with higher concentrations of serum having a greater effect on inhibition of both CD16 down-modulation (Figure 6A) and CD54 upregulation (Figure 6B). When the serum was heat-inactivated, the inhibitory effect of the serum was abrogated. These results demonstrate that the inhibitory

properties of serum are dose dependent and heat-labile, suggesting that the inhibition is due to complement activity.

Inhibitory effects of complement activity are not influenced by target cell viability

As would be expected, the addition of serum and rituximab to Raji cells induced lysis of the targets both in the presence and absence of PBMCs (Fig. 7). This cell death, most likely due to CDC, was observed at rituximab concentrations beginning at 0.04μg/ml. As described above, NK cell CD54 peaks and begins to decrease at 0.04μg/ml rituximab when the co-cultures are performed in the presence of serum. This suggests that lysis of rituximab-coated target cells through CDC could be inhibiting the ability of these targets to activate the NK cells. In order to evaluate this possibility, I assessed whether target cell viability affects the ability of rituximab to induce NK cell CD16 down-modulation. Raji cells were fixed in 1% formaldehyde and washed before being used in the co-culture assay. Results with fixed Raji cells were similar to those seen with viable target cells. In the absence of serum, surface CD16 on NK cells was modulated by rituximab-coated fixed Raji cells. This down-modulation was blocked when serum was added (Figure 8A). To confirm that the inhibition observed was independent of target cell viability, PBMCs were added to culture plates coated directly with rituximab in the absence of target cells. Under these conditions, NK cell CD16 was down-modulated in the media alone, while the addition of serum blocked this down-modulation (Figure 8B). These studies utilizing systems free of viable target cells indicate that the inhibitory effect of serum on rituximab-induced CD16 down-modulation is not due to lysis of the target cells by CDC.

C1q and C3 are necessary for the inhibitory effects of complement activity

To confirm that the inhibitory activity of serum is due to complement, activation of NK cells induced by rituximab-coated target cells was assessed in the presence of serum depleted of C1q, C3 or C5. The addition of C1q-depleted serum to the co-culture failed to inhibit NK cell CD54 upregulation induced by rituximab-coated targets (Figure 9A). The inhibition of NK cell CD54 upregulation was partially restored when purified C1q was added to the C1q depleted serum. Purified C1q in the absence of serum had no effect on NK cell CD54 expression. Similar results were observed in experiments using C3-depleted serum. Serum depleted of C3 failed to inhibit NK cell CD54 upregulation. However, the inhibitory effect of serum was partially restored when purified C3 was added to the C3 depleted serum (Figure 9B). Because the concentration of C3 in normal human serum ranges from 1-2 mg/ml, the amount of C3 used was titrated to 2 mg/ml. Purified C3 also had no effect on NK cell CD54 expression in the absence of serum. In contrast, serum depleted of C5 successfully blocked NK cell CD54 upregulation induced by rituximab-coated Raji cells (Figure 9C). In addition, there was no evidence of target cell lysis through CDC in the presence of C5 depleted serum (data not shown). The finding that C3, but not C5 is necessary to block rituximab-induced NK activation suggests that C3b deposition on the rituximab-coated targets is the key component in the observed inhibitory effect.

Inhibition of NK cell activation correlates with C3b deposition

It was previously reported that mAb 3E7 prevents the degradation of C3b by competing with factor H and factor I, thereby increasing the amount of C3b present on the surface of target cells(154). We used this C3b stabilizing mAb to further correlate C3 with the inhibition of NK cell activation. The addition of 3E7 to serum in the co-culture

assay enhanced the degree of inhibition of NK cell CD54 upregulation when compared to human serum alone (Figure 10). These results suggest increased amounts of C3b on the surface of targets enhance the inhibitory effects of serum and provide evidence that C3b is responsible for the inhibition of NK cell activation induced by rituximab-coated Raji cells.

Blocking CR3 does not affect inhibition of NK activation

C3b is a ligand for complement receptor 3 (CR3, Mac-1, CD11b/CD18) which is expressed by NK cells. One explanation for the findings outlined above is that C3b induces an NK cell inhibitory signal via CR3, or alternatively that it blocks a CR3 activation signal from a different ligand. CR3 blocking mAbs have been shown to inhibit CR3 mediated signaling and phagocytosis of effector cells (155-157). I utilized blocking mAbs to CD11b and CD18 to investigate whether the ability of complement to inhibit NK activation is through CR3. The inhibitory effects of human serum on rituximab-induced NK cell CD16 down-modulation and CD54 upregulation continued to be observed in the presence of anti-CD11b and anti-CD18 antibodies (Figure 11). This suggests the inhibition of NK activation mediated by complement is not mediated through CR3.

Complement activity inhibits the binding of NK cell CD16 to rituximab

Because C3b deposits on human Fc regions that interact with CD16, it is possible that the activation of complement affects the binding of NK cells to rituximab. In order to further explore the mechanism in which complement fixation inhibits NK cell activation, a cell adhesion assay was developed to analyze the effect of complement on the avidity between CD16 of intact NK cells and human IgG. Purified NK cells were applied to U-bottomed plates, centrifuged after incubation, and analyzed for absorbance at 405nm. In the absence of rituximab, the cells pelleted at the bottom of the wells as shown by a high

absorbance. When the plate was coated with rituximab, NK cells adhered to the walls and prevented the formation of a pellet, resulting in lower absorbance. An anti-CD16 mAb blocked this adhesion while an isotype control did not, indicating that the NK cell adherence is through its CD16 binding to rituximab (data not shown). When the rituximab-coated plates were incubated with serum prior to application of NK cells, the adherence was blocked (Figure 12). Heat-inactivated serum was less effective at blocking the binding of NK cells to the walls of the plate. The residual inhibition of the heat-inactivated serum is likely due to IgG within the serum binding to Fc receptors on the NK cells. These results provide further evidence that complement fixation blocks the binding of CD16 on NK cells to rituximab.

Complement activity inhibits NK cell-mediated cytotoxicity

We next evaluated whether complement impairs rituximab-mediated ADCC using purified NK cells as effector cells. These studies were done using C5-depleted serum because such serum allows for activation of the earlier steps in the complement cascade, including C3b deposition, but does not induce development of the membrane attack complex or CDC of the target cell. As expected, C5-depleted serum was unable to induce CDC of rituximab-coated Raji cells (data not shown). Also as expected, purified NK cells induced ADCC of rituximab-coated Raji cells in the absence of serum. ADCC was inhibited by C5-depleted serum, but not by heat-inactivated, C5-depleted serum (Figure 13). These studies demonstrate that complement fixation upstream of C5 activation impairs the lysis of rituximab-coated target cells by NK cells.

Discussion

Most in vitro studies on mAb mechanisms of action involve the use of cytotoxicity assays. Measurements of cell lysis, when mAbs are added to target cells in the absence of immune effector mechanisms, are widely used to study signaling induced

apoptosis. Serum is included as a source of complement in cytotoxicity assays involving mAbs and target cells to study CDC. ADCC is usually studied in the absence of human serum through the quantitation of cell lysis by the addition of effector cells to mAbs and target cells. These assays have proven to be quite informative when individual mechanisms are under evaluation. However, measuring target lysis alone makes it difficult to assess the relative role of each mechanism under physiologic conditions in which target cells, effector cells, complement, and the mAb are all present. In the studies outlined above, I evaluated how complement impacts on NK cell activation induced by mAb-coated target cells. Malignant B cell lines served as the target cell while rituximab was used as the mAb. This assay allowed us to quantitate NK activation separately from mAb induced target lysis, which results from a variety of mechanisms.

These studies demonstrated that serum, but not heat-inactivated serum, blocks activation of NK cells by rituximab-coated target cells. The effect of serum on CD16 down-modulation was seen with non-viable target cells and in a target cell-free system, indicating that the inhibition of NK activation was not due to rapid CDC of target cells. Instead, it appears blocking of rituximab-mediated signaling via CD16 is likely responsible for this effect. C1 and C3 were required for this inhibition, but not C5. The ability of a C3b stabilizing mAb to enhance the blocking effect points towards C3b deposition as being responsible. An adhesion assay demonstrated that serum effectively interferes with the binding of NK cell CD16 to rituximab. C5-depleted serum blocked NK-mediated ADCC, indicating early steps in the complement cascade not only inhibit NK activation, but also inhibit NK-mediated lysis of mAb-coated targets.

These results provide further evidence that CDC and ADCC are not completely independent mechanisms. Complement activation can deposit C3b (and its subsequent breakdown products iC3b and C3dg) on mAb-opsonized cells. Both C3b and iC3b can act as opsonins to enhance cell-mediated killing, even if the cells are not directly lysed by the membrane attack complex (158). Moreover, activation of complement by IgG-

opsonized substrates can generate C5a, which can serve as a chemotactic factor to attract inflammatory cells. C5a also acts to upregulate both CR3 (159, 160), and activating FcγRIII on monocytes and macrophages, thus enhancing local inflammation and ADCC at the tumor site (135, 161-163).

Activation of NK cells via CD16 may be mediated, at least in part, by interactions involving the CD11b/CD18 (CR3) molecules on the surface of the NK cell (164). C3b binding to CR3 could interfere with this interaction. As outlined above, serum inhibited NK activation even in the presence of antibodies that block CD11b and CD18 suggesting the observed effects on rituximab-induced NK activation are not due solely to a CR3-mediated pathway. Hong et al have reported beta1,3-glucans can interact with CR3 and enhance sensitivity of antibody-coated target cells to immune effectors (165, 166). Indeed, the relationship between CD16, IgG Fc, C3b and CR3 appears to be quite complex, and the blocking studies outlined above do not totally exclude the possibility that CR3 is playing a role in the observed inhibition of NK activation by complement. Further studies are needed to explore how these interactions impact on CR3 pathway signaling.

An adherence assay was developed to assess the effect of complement on the interactions between CD16 on intact cells and IgG. The cell-adhesion approach was used because it allowed us to use live NK cells to study CD16 binding in its natural conformation in the membrane. Importantly, this assay was done in the absence of target cells. Therefore, it only measured interactions of the NK cell with the IgG, and would not be influenced by adhesion interactions between NK cells and target cells. Using this assay, I demonstrated that complement fixation blocks the binding of CD16 on the NK cells to rituximab. This finding is consistent with the known structures of these molecules. Crystal structures demonstrate that CD16 binds to human IgG1 Fc in the area of the Cγ2 and hinge region (68). The Cγ2 and hinge region has also been shown to be where C3b binds to human IgG1 Fc (167). Given that C3b is a large (177kDa) protein,

C3b deposited on the Fc portion of IgG1 could well hinder the interaction between the mAb and CD16. These results provide further evidence that a primary mechanism involved in the observations outlined above is the ability of C3b to block interaction between CD16 and the Fc portion of human IgG1.

The ability to induce complement fixation varies depending on the mAb. Different anti-CD20 mAbs have been shown to vary in their ability to mediate CDC (30, 168, 169). In addition, trastuzumab is not efficient at fixing complement and the data on the ability of alemtuzumab, an anti-CD52 mAb, to induce CDC is conflicting (32, 33). Further studies are needed to explore whether serum inhibits the ability of each of these mAb to induce NK activation and mediate ADCC. Preliminary results indicate the inhibition of NK cell activation by complement is not observed with all mAbs. For example, in contrast to the results with rituximab presented above, it has been found that serum has no effect on the ability of trastuzumab on breast cancer cells to activate NK cells. Further studies are needed to explore whether a correlation exists between the ability of a mAb to mediate CDC and the inhibition of its ability to activate NK cells and mediate ADCC in the presence of serum.

There is conflicting evidence as to whether levels of complement regulatory proteins CD55 and CD59 play a role in clinical response to anti-cancer mAbs including rituximab. In vitro studies demonstrate a correlation between low CD55 and CD59 and greater CDC induced lysis of target cells (25, 26). However, no correlation has been found between expression of these proteins by malignant B cells and response to rituximab therapy (29). These findings suggest a possible explanation for this discrepancy. CD59 blocks the membrane attack complex, but has no influence on C3b opsonization. However, complement control protein CD55 suppresses complement activation at the C3b activation stage (170, 171). Thus, higher CD55 expression could suppress C3b deposition, thereby paradoxically allowing for more effective NK activation and ADCC. In other words, elevated CD55 activity could lead to a decrease in

CDC, but allow for enhanced NK activation and ADCC. Further studies are needed to explore this possibility by assessing whether higher CD55 expression leads to more effective NK activation in the assays outlined above.

NK activation in the absence of serum is first seen at mAb concentrations of approximately 0.002μg/ml. In contrast, a concentration of approximately 0.04μg/ml is required for CDC to occur. The concentration in which mAb begins to block NK activation is similar to the concentration of mAb that induces CDC (0.04 μg/ml). This is consistent with both CDC and blockade of NK activation being dependent on initiation of the complement cascade. NK activation is likely induced when multiple mAb molecules form a bridge between the target and effector cells in the immunologic synapse, and so cross-link CD16. This occurs at a relatively low mAb concentration because of the interactions between CD16 and multiple Fc molecules. In contrast, complement interacts with individual or a small number of mAb molecules on the surface of the target cell. As illustrated in this chapter, higher concentrations of mAb are need to activate complement to induce either CDC or block NK activation. The observation that NK activation begins at a lower mAb concentration than does the inhibitory effect of complement fixation could have clinical implications. There appears to be a window of mAb concentrations between 0.002 μg/ml and 0.04 μg/ml in which NK activation is induced without fixation of complement. Serum concentrations of mAb in patients during rituximab therapy may range up to 502.8 ug/ml, which is much higher than the concentration needed to activate complement (11). This suggests that a more precise titration of mAb levels to fall within this window could lead to greater ADCC.

Our current understanding of the structure-function relationships of the mAb Fc allows us to engineer mAb with modified Fc function. Most effort in this regard has been geared towards enhancing affinity of mAb Fc for Fcγ receptors (91). However, it is also possible to design a mAb that continues to recognize FcγR but no longer fixes complement. It has been shown that alanine substations at positions D270, K322, P329,

and P331 significantly reduce the ability of rituximab to bind C1q and activate complement (48). The results outlined above and the data supporting ADCC as a central mechanism of action, suggest that an IgG which activates NK cells without fixing complement may widen the window and be more effective therapeutically by mediating ADCC at high mAb concentrations.

In conclusion, this chapter demonstrates that complement fixation resulting in C3b deposition inhibits the ability of mAb-coated targets to activate NK cells. This is most likely due to C3b blocking the interaction between the Fc portion of rituximab and CD16 (Figure 12). We know CDC can serve as a mechanism by which target cells can be killed. These studies suggest complement fixation can also have an inhibitory effect on NK activation and ultimately ADCC. Growing evidence indicates that ADCC is crucial to the anti-tumor effect of mAb therapy. If ADCC indeed serves as a major mechanism in mAb therapy, the ability of mAb to fix complement may actually limit its clinical efficacy. These findings, if confirmed, could have significant implications on how we use available mAbs, and how we prioritize selection of mAb with engineered function for future development.

Figure 5. Serum inhibits rituximab-induced NK cell CD16 down-modulation and CD54 upregulation.

PBMCs and Raji, Daudi, or FL cells were mixed at a 1:1 ratio for 20h in the presence or absence of 50% serum with varying concentrations of rituximab. Surface marker expression was determined using flow cytometry with gating on CD3-, CD56+ lymphocytes. (A) NK cell CD16, expressed as median fluorescence, in the absence and presence of serum (n=3). (B) NK cell CD54, expressed as percent CD54 bright, in the absence and presence of serum (n=3). The error bars represent the standard deviation of the mean. Statistical difference ($P<0.05$) between media and serum was noted at rituximab concentrations greater than 0.2μg/ml by paired student t-test.

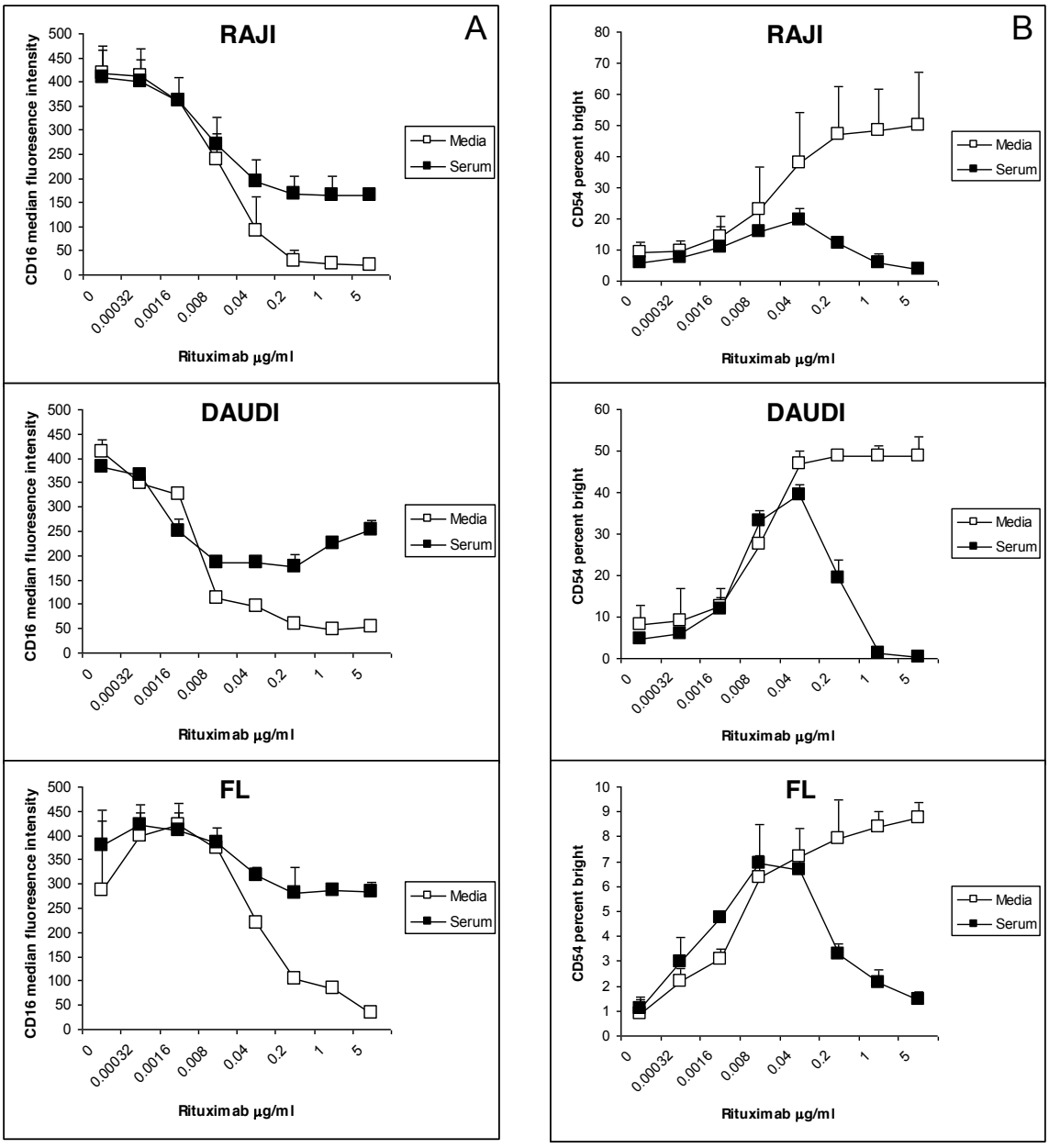
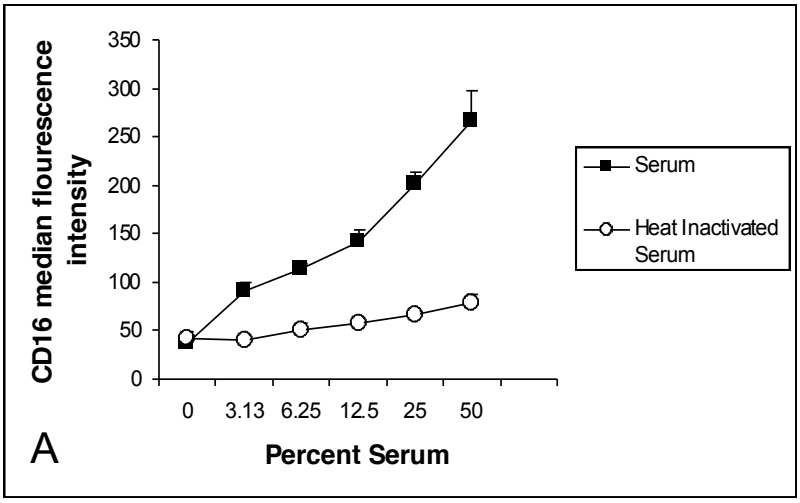

A
RAJI
CD16 median fluoresence intensity
Rituximab µg/ml
Media
Serum
DAUDI
FL
B
CD54 percent bright
0
0.00032
0.0016
0.008
0.04
0.2
1
5

Figure 6. Inhibitory effect of serum is dose dependent and abrogated by heat inactivation.

PBMCs and Raji were mixed at a 1:1 ratio for 20h in varying concentrations of serum or heat inactivated serum in the presence of 0.2μg/ml of rituximab. Surface marker expression was determined using flow cytometry with gating on CD3-, CD56+ lymphocytes. (A) NK cell CD16, expressed as median fluorescence, after incubation with varying concentrations of serum and heat inactivated serum (n=3). (B) NK cell CD54, expressed as percent CD54 bright, after incubation with varying concentrations of serum and heat inactivated serum (n=3). The error bars represent the standard deviation of the mean. Statistical difference ($P<0.05$) between serum and heat inactivated serum was noted at concentrations greater than 3.124% by paired student t-test.


CD16 median flourescence intensity
350
300
250
200
150
100
50
0
0
3.13
6.25
12.5
25
50
Percent Serum
Serum
Heat Inactivated Serum
A

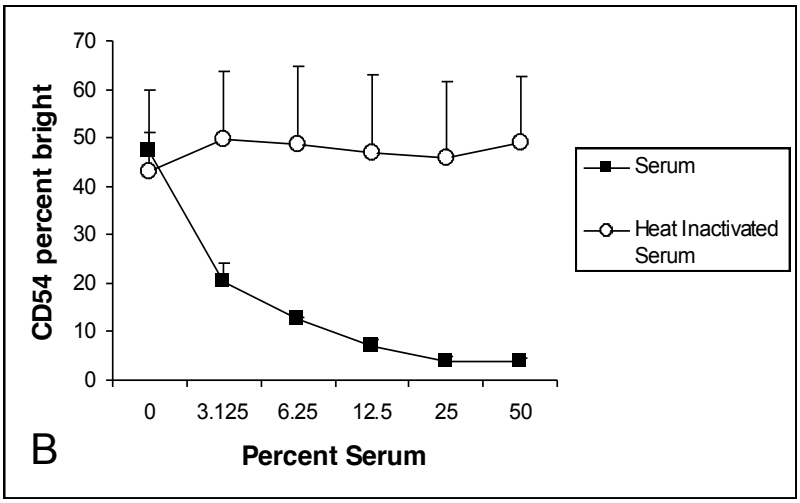

CD54 percent bright
70
60
50
40
30
20
10
0
0
3.125
6.25
12.5
25
50
Percent Serum
Serum
Heat Inactivated Serum
B

Figure 7. Target cells are lysed in the presence of serum and rituximab irrespective of presence of effector cells.

Raji were incubated with PBMCs at a 1:1 ratio for 20h in media, 50% serum, or 50% serum without PBMCs with varying concentrations of rituximab. The percent of viable target cells were determined using flow cytometry by counting annexin V and propidium iodide negative target cells (n=3). The error bars represent the standard deviation of the mean. Statistical difference ($P<0.05$) between media and serum with or without PBMCs was noted at rituximab concentrations greater than 0.04μg/ml by paired student t-test.

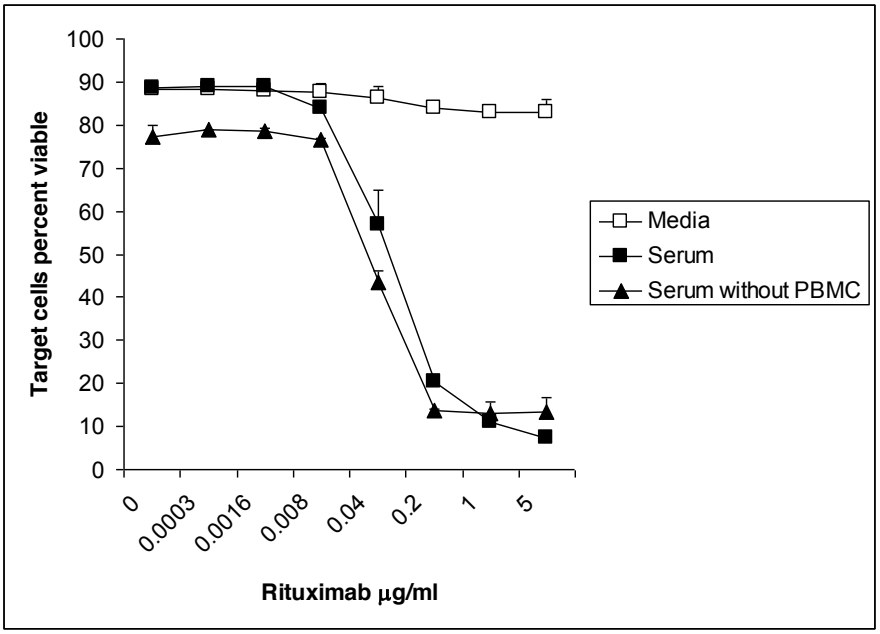
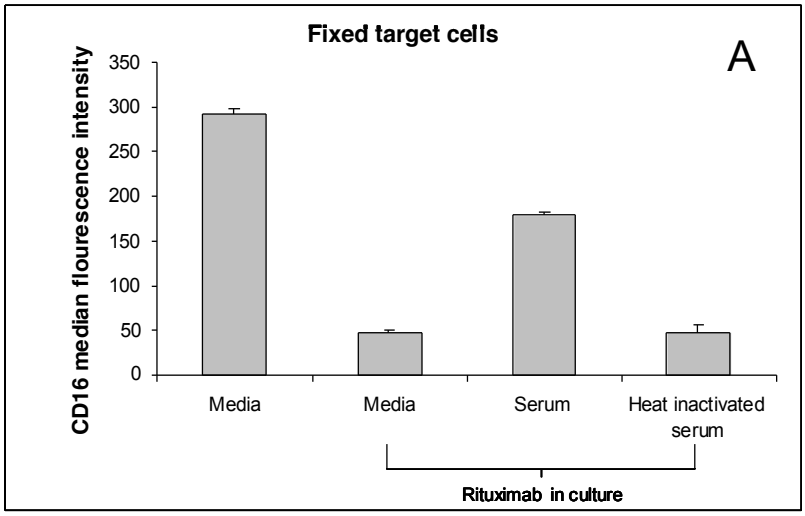

100
90
80
70
60
50
40
30
20
10
0
Target cells percent viable
0
0.0003
0.0016
0.008
0.04
0.2
1
5
Rituximab μg/ml
Media
Serum
Serum without PBMC

Figure 8. Inhibitory effect of serum on CD16 expression occurs in the absence of viable target cells.

(A) Raji cells were fixed in 1% formaldehyde and washed extensively. Fixed cells were mixed with PBMCs at a 1:1 ratio for 20h with 5μg/ml of rituximab (n=3). (B) Flat bottom plates coated with 10μg/ml of rituximab. Plates were washed, and PBMCs were added and cultured for 20h. Incubations with fixed Raji cells and rituximab or rituximab-coated plates were preformed in the presence or absence of 50% serum and heat inactivated serum (n=5). Surface marker expression was determined using flow cytometry with gating on CD3-, CD56+ lymphocytes. NK cell CD16, expressed as median fluorescence, was measured after incubation with media, serum and heat inactivated serum in the presence of fixed Raji cells (A) or flat bottom plates coated with rituximab (B). The error bars represent the standard deviation of the mean. Statistical difference ($P<0.05$) between media and serum was noted when rituximab was present by paired student t-test.


Fixed target cells
A
CD16 median flourescence intensity
350
300
250
200
150
100
50
0
Media
Media
Serum
Heat inactivated serum
Rituximab in culture

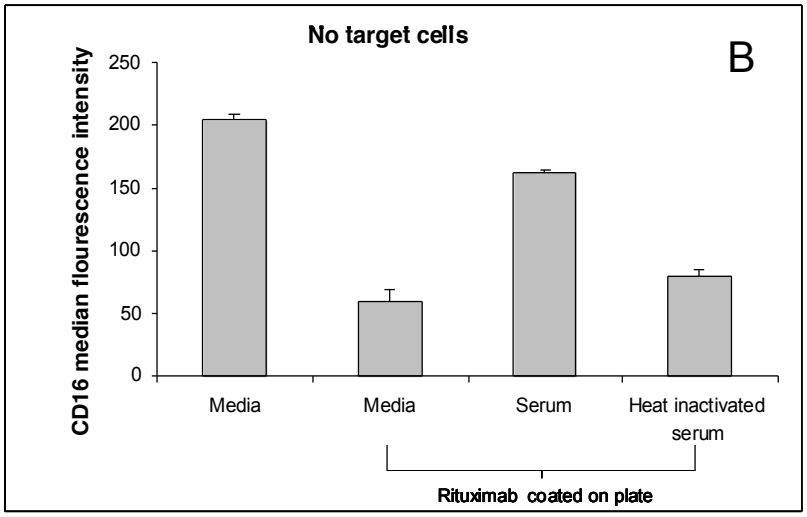
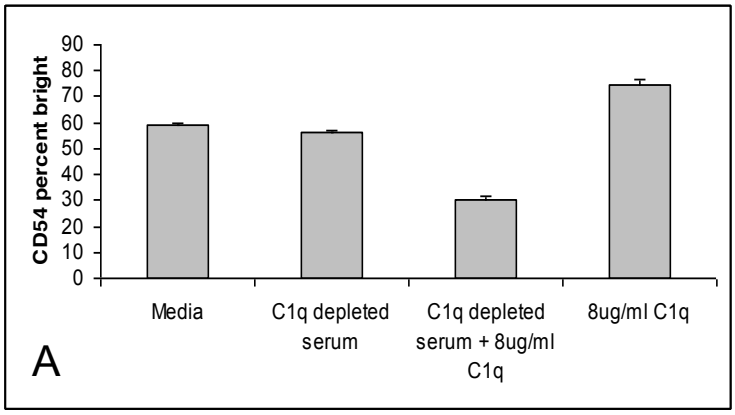
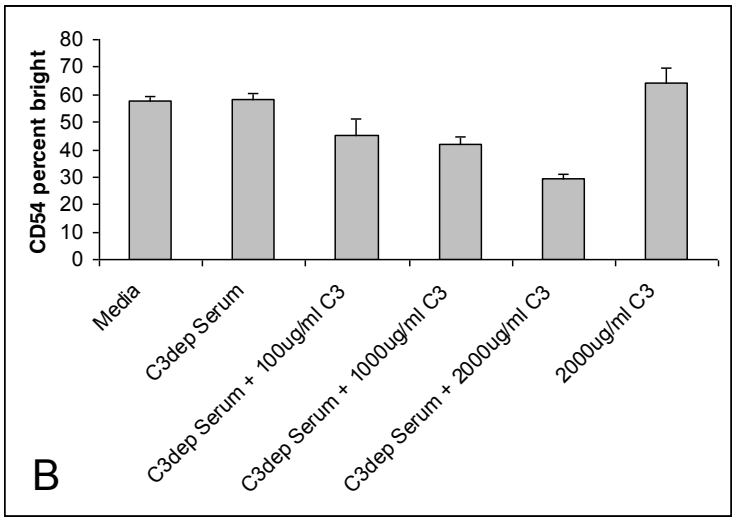

No target cells
B
CD16 median flourescence intensity
250
200
150
100
50
0
Media
Media
Serum
Heat inactivated serum
Rituximab coated on plate

Figure 9. Serum depleted of C5 inhibits NK cell CD54 upregulation while serum depleted of C1q or C3 does not.

PBMCs were mixed with Raji at a 1:1 ratio for 20h with 5μg/ml of rituximab in the presence or absence of C1q, C3, or C5 depleted serum. Surface marker expression was determined using flow cytometry with gating on CD3-, CD56+ lymphocytes. NK cell CD54, expressed as percent CD54 bright, was determined. (A) Evaluation in C1q depleted serum, C1q depleted serum with purified C1q added back, or purified C1q alone (n=3). Statistical difference (P=0.0013) between media and C1q depleted serum + 8μg/ml C1q was noted by paired student t-test. (B) Evaluation in C3 depleted serum, C3 depleted serum with purified C3 added back, or purified C3 alone (n=3). Statistical difference (P=0.0028) between media and C3 depleted serum + 2000μg/ml C3 was noted by paired student t-test. (C) Evaluation in various concentrations of C5 depleted serum (n=3). Statistical difference ($P<0.05$) between media and C5 depleted serum was noted at all concentrations by paired student t-test. The error bars represent the standard deviation of the mean.


CD54 percent bright
90
80
70
60
50
40
30
20
10
0
Media
C1q depleted serum
C1q depleted serum + 8ug/ml C1q
8ug/ml C1q
A


CD54 percent bright
80
70
60
50
40
30
20
10
0
Media
C3dep Serum
C3dep Serum + 100ug/ml C3
C3dep Serum + 1000ug/ml C3
C3dep Serum + 2000ug/ml C3
2000ug/ml C3
B

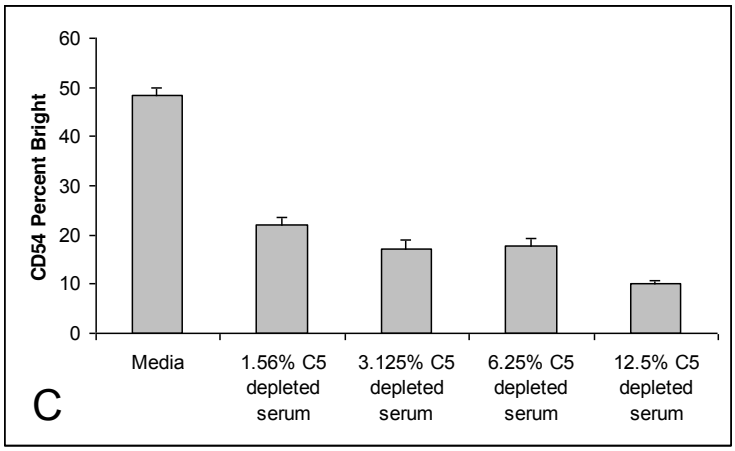

CD54 Percent Bright
60
50
40
30
20
10
0
Media
1.56% C5 depleted serum
3.125% C5 depleted serum
6.25% C5 depleted serum
12.5% C5 depleted serum
C

Figure 10. C3b stabilizing antibody (3E7) enhances the inhibition of NK cell CD16 down-modulation and CD54 upregulation.

PBMCs and Raji were mixed at a 1:1 ratio for 20h in the presence or absence of 50% serum with varying concentrations of rituximab and 10μg/ml of 3E7. Surface marker expression was determined using flow cytometry with gating on CD3-, CD56+ lymphocytes. NK cell CD54, expressed as percent CD54 bright, cultured in media, serum or serum plus 3E7 (n=3). The error bars represent the standard deviation of the mean. Statistical difference (P=0.0088) between serum and serum + 3E7 at 5μg/ml of rituximab was noted by paired student t-test.

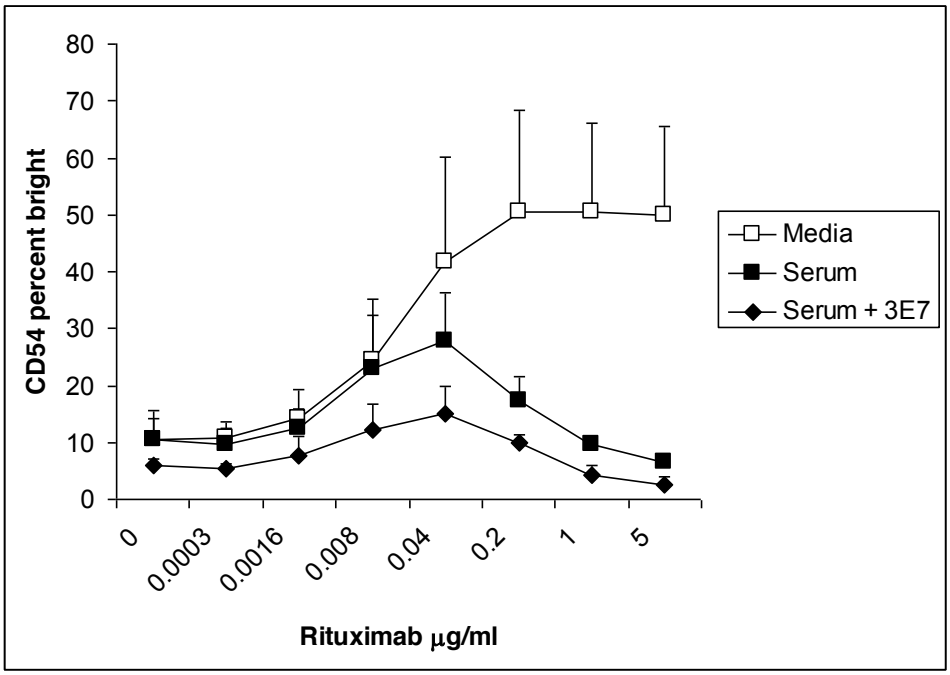
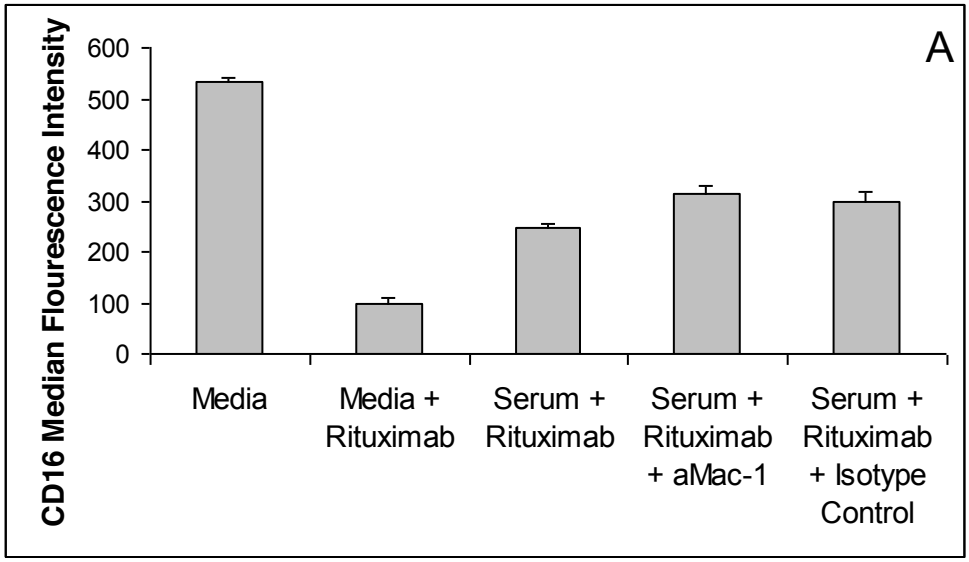

80
70
60
50
40
30
20
10
0
CD54 percent bright
0
0.0003
0.0016
0.008
0.04
0.2
1
5
Rituximab μg/ml
Media
Serum
Serum + 3E7

Figure 11. Blocking CR3 does not affect inhibition of NK activation.

PBMCs were mixed with Raji at a 1:1 ratio for 20h with 5μg/ml of rituximab in the presence or absence of 50% serum, 25μg/ml anti-CD11b mAb and 25μg/ml anti-CD18 mAb (anti-Mac-1), or isotype control. Surface marker expression was determined using flow cytometry with gating on CD3-, CD56+ lymphocytes. (A) NK cell CD16, expressed as median fluorescence, in the absence or presence of serum, anti-Mac-1 mAb, or isotype control (n=3). (B) NK cell CD54 expressed as percent CD54 bright, in the absence or presence of serum, anti-Mac-1 mAb, or isotype control (n=3). The error bars represent the standard deviation of the mean.


A
CD16 Median Flourescence Intensity
600
500
400
300
200
100
0
Media
Media + Rituximab
Serum + Rituximab
Serum + Rituximab + aMac-1
Serum + Rituximab + Isotype Control

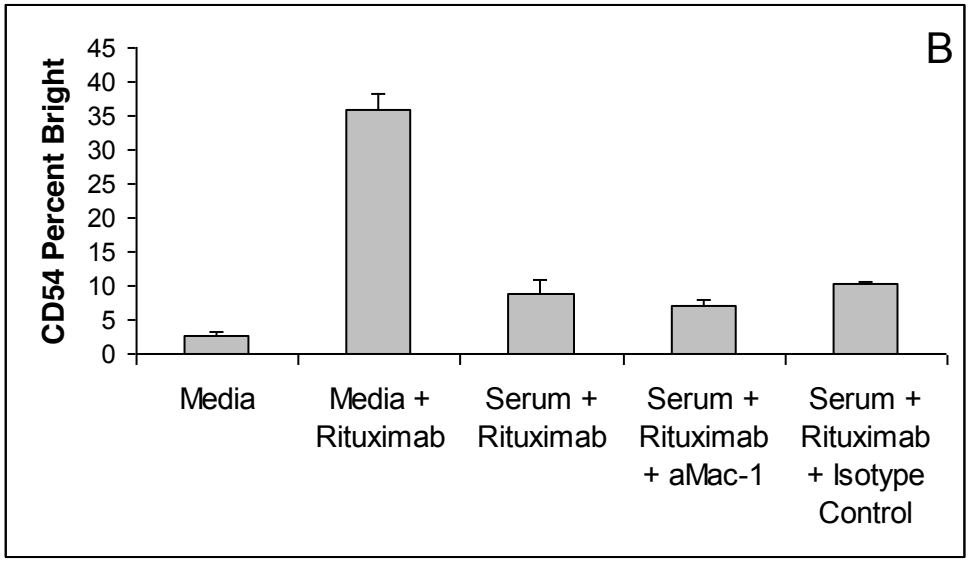

B
CD54 Percent Bright
45
40
35
30
25
20
15
10
5
0
Media
Media + Rituximab
Serum + Rituximab
Serum + Rituximab + aMac-1
Serum + Rituximab + Isotype Control

Figure 12. NK cell CD16 binding to rituximab is blocked by complement fixation.

U-bottomed 96-well microtiter plates were coated with varying concentrations of rituximab. Wells were incubated with media, 50% serum, or 50% heat-inactivated serum. After washing, NK cells were allowed to sit on plate for 30 min at room temperature. High absorbance resulted from NK cell pelleting that occurred in the absence of binding to rituximab. All samples were run in triplicate (n=3). This figure is a representative of 3 independent experiments. The error bars represent the standard deviation of the mean. Statistical difference ($P<0.05$) between media and serum was noted at rituximab concentrations greater than 5μg/ml by paired student t-test.

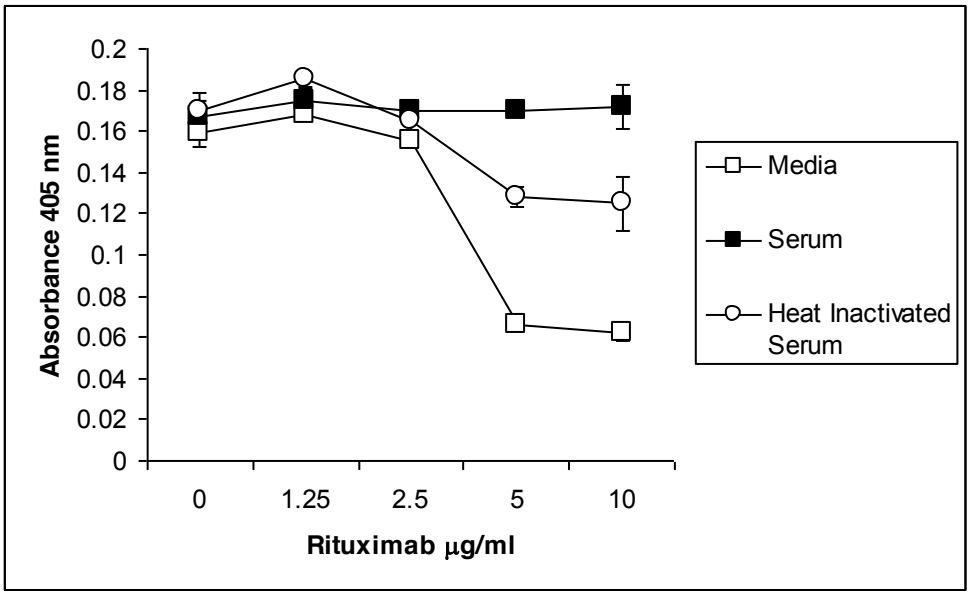

0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Absorbance 405 nm
0
1.25
2.5
5
10
Rituximab μg/ml
Media
Serum
Heat Inactivated Serum

Figure 13. C5-depleted serum inhibits rituximab-mediated ADCC.

Raji cells were labelled with ^{51}Cr and incubated with purified NK cells in various E:T ratios, 5μg/ml of rituximab, and either C5-depleted serum, heat-inactivated C5-depleted serum or media. Percent specific lysis was measured based on ^{51}Cr release. All samples were run in triplicate (n=3). This figure is a representative of three independent experiments. The error bars represent the standard deviation of the mean. Statistical difference ($P<0.05$) between media and C5-depleted serum was noted at E:T ratios greater than 6.25:1 by paired student t-test.

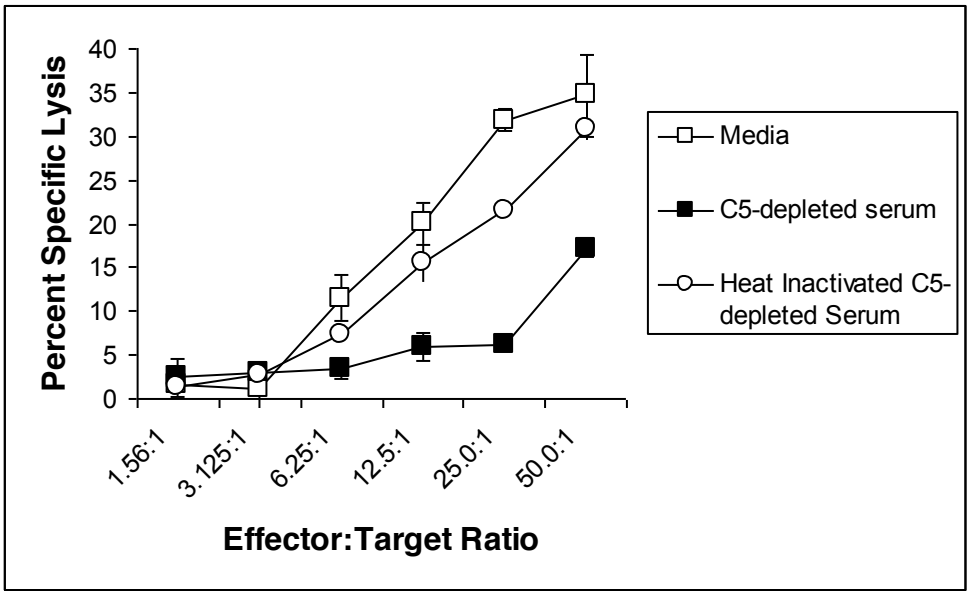
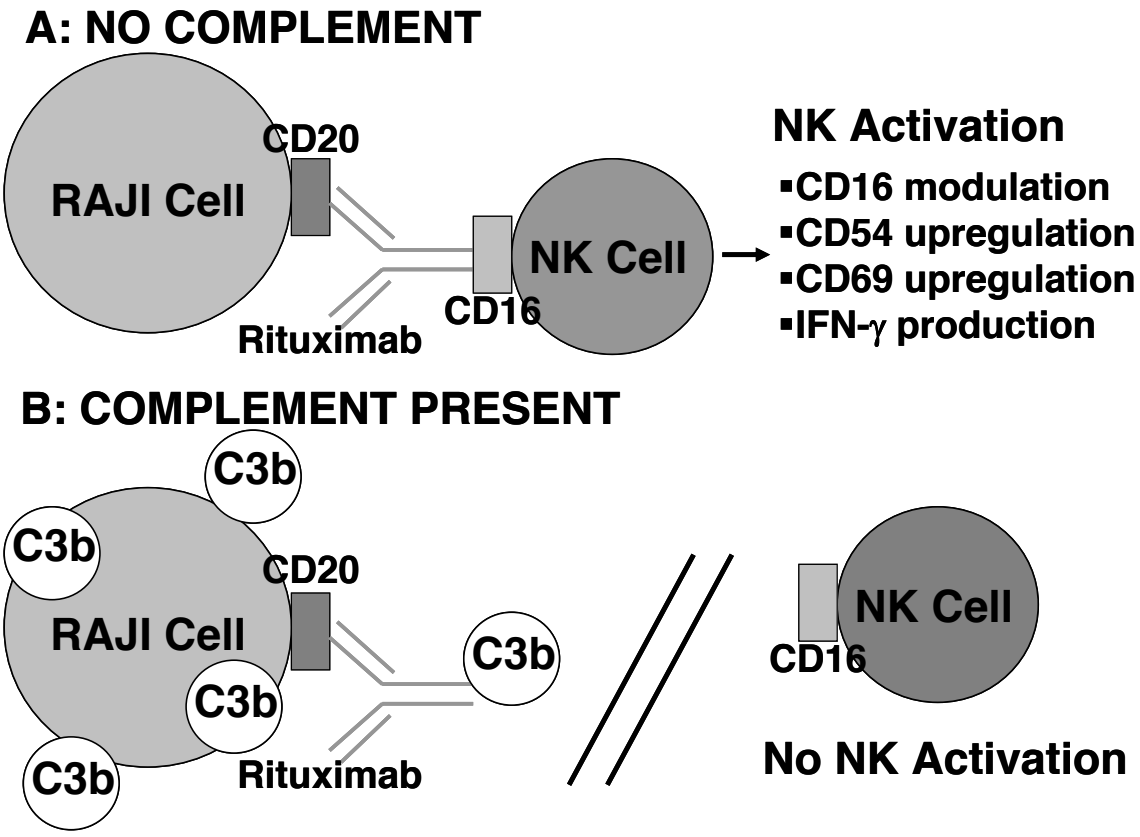

Percent Specific Lysis
40
35
30
25
20
15
10
5
0
1.56:1
3.125:1
6.25:1
12.5:1
25.0:1
50.0:1
Effector:Target Ratio
Media
C5-depleted serum
Heat Inactivated C5-depleted Serum

Figure 14. Schematic depiction of the interaction between complement fixation and NK cell activation.

(A) In the absence of complement, NK cell CD16 binds to the Fc portion of rituximab. Signaling leads to the activation of NK cells. (B) In the presence of complement, C3b deposition on rituximab-coated targets prevents the binding of NK cell CD16 to the Fc portion of rituximab. The lack of binding prevents the activation of NK cells.


A: NO COMPLEMENT
RAJI Cell
CD20
Rituximab
CD16
NK Cell
NK Activation
▪CD16 modulation
▪CD54 upregulation
▪CD69 upregulation
▪IFN-γ production
B: COMPLEMENT PRESENT
C3b
C3b
C3b
C3b
C3b
RAJI Cell
CD20
Rituximab
CD16
NK Cell
No NK Activation

CHAPTER III

DEPLETION OF COMPLEMENT ACTIVITY ENHANCES NK CELL ACTIVATION AND IMPROVES THE EFFICACY OF A MONOCLONAL ANTIBODY THERAPY IN VIVO

Abstract

Antibody-dependent cellular cytotoxicity (ADCC) is felt to play an important role in mediating the anti-tumor effects of rituximab. It was previously reported that C3b deposition inhibits NK cell activation and ADCC induced by rituximab-coated targets. We evaluated whether depletion of C3 enhances the ability of mAb-coated targets to activate NK cells in vitro and improves mAb therapy in vivo. Normal human serum inhibited the ability of rituximab-coated lymphoma cells to activate NK cells as previously reported. NK activation was increased when serum was pre-incubated with cobra venom factor (CVF) to deplete C3. Similar results were found when transudative pleural fluid or non-malignant ascites, as a surrogate for extravascular fluid, was used as the source of complement. For in vivo analysis, we depleted C3 in a syngeneic, immunocompetent murine model previously used to study ADCC. Treatment with an anti-idiotype mAb (MS11G6) prolonged the survival of C3H/HeN mice inoculated with murine 38C13 lymphoma cells. Survival following treatment with CVF plus mAb and a human C3 derivative with CVF-like functions (HC3-1496) plus mAb were both superior to that of mAb alone. These studies demonstrate that complement depletion enhances NK cell activation induced by rituximab-coated targets and improves the efficacy of mAb therapy in a murine lymphoma model. Furthermore, these studies suggest that inhibitory effect of complement may be present in the extravascular compartment, in which many malignant lymphocytes reside.

Introduction

Monoclonal antibody (mAb)-based therapies are now standard treatment for various malignancies. The chimeric anti-CD20 mAb, rituximab, remains the gold standard with respect to clinically effective mAbs. Antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) both have been shown to contribute to the anti-tumor activity of mAbs in preclinical models. However, their relative importance in the clinical efficacy of rituximab and other mAbs remain unclear.

Data from both laboratory models and correlative clinical studies suggest that ADCC plays a significant role in the anti-tumor effects of mAbs. Clynes et al showed that the therapeutic effect of mAbs is lost in Fcγ-receptor knock-out mice (76, 77). In clinical investigations, three independent studies demonstrated that single agent rituximab is more effective in patients with Fcγ receptor III (CD16) polymorphisms associated with higher affinities for human IgG. Patients homozygous for the V158 polymorphism (VV) on CD16 have higher clinical response rates to rituximab than patients that are carriers for F158 (VF or FF), suggesting that Fc receptors on effector cells play a key role in the therapeutic effect of rituximab (80-82).

Rituximab has also been demonstrated by in vitro studies to be highly efficient in mediating CDC of various B-cell lines as well as fresh samples (17, 27-29). Several in vivo tumor models suggest that the anti-tumor activity of rituximab is dependent, at least in part, on complement (30, 36, 38). In addition, clinical observations provide evidence that complement is activated during treatment with rituximab (172). In a small study, complement activation was found to correlate with the infusional toxicity often seen in patients with high numbers of circulating B cells (40). However, it is unclear whether this is a causative relationship. Recently, Tawara and colleagues reported that complement activation plays a key role in the antibody-induced infusion toxicity of mAbs in animal models. These studies demonstrated that modified mAbs with limited complement fixing

ability resulted in reduced infusion reactions. However, the lack of complement activation did not affect the anti-tumor activity (173). In addition, a clinical study found that expression levels of complement inhibitors failed to predict the clinical outcome of rituximab treatment (29). Although there is solid laboratory evidence that complement may be important for the anti-tumor effect of mAbs, the clinical evidence is less clear.

Previously, an in vitro assay was developed that measures mAb-induced NK activation through assessing NK cell surface phenotypes (91). This system was used to evaluate the relationship between complement fixation and the ability of rituximab-coated targets to induce NK cell activation. It was found that complement deposition interferes with the binding of NK cells to rituximab, preventing the activation of NK cells as measured by the down-modulation of CD16 and the upregulation of the activation markers, CD54 and CD69. This inhibition was dependent on the presence of C1q and C3 but not C5. NK cell mediated lysis of rituximab-coated target cells was also inhibited by complement activation (174). These results suggest that if ADCC is indeed the central mechanism of action, complement activation may actually be limiting the therapeutic effect of rituximab.

These findings are in contrast to the traditional assumption that complement activation contributes to the efficacy of rituximab. However, several important questions related to this hypothesis remain unanswered. As with most complement studies, normal human serum was used as the source of complement. It is unclear whether the complement proteins present in serum are active within extravascular sites, in which many of the non-circulating malignant lymphocytes reside. In this chapter, I utilized transudative pleural fluid and non-malignant ascites as surrogates for extravascular fluid to determine whether the inhibitory effects of complement are clinically relevant. I also evaluated the effect of complement depletion on the ability of rituximab-coated target cells to activate NK cells and on the efficacy of a mAb in a model previously used to study ADCC. I found that transudative pleural fluid and non-malignant ascites inhibited

NK cell activation induced by rituximab-coated targets. The use of complement depleting agents enhanced NK cell activation in vitro and improved the efficacy of a mAb therapy in vivo. These results suggest that complement activity in both the circulation and the extravascular compartment may be inhibiting rituximab-induced NK cell activation, and that limiting complement activity may be an appropriate strategy to enhance the efficacy of mAbs.

Materials and methods

Antibodies and reagents

Rituximab (Biogen-Idec, Cambridge, MA; and Genentech, South San Francisco, CA) was purchased commercially. The therapeutic murine mAb, MS11G6 (IgG2a), was obtained from tissue culture supernatant by protein A affinity chromatography (175). CVF was was prepared from lyophilized venom from *N. naja* (176). HC3-1496 is a human C3/CVF hybrid protein containing a 168 amino acid residue substitution of CVF sequence at the C-terminus of the α-chain of C3 (177).

Samples from human subjects

Informed consent from subjects was obtained prior to use of all human samples. Normal human serum and peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers and processed and analyzed (91). Mononuclear cells were isolated and red blood cells were removed by resuspending cells in 5 mL red cell lysis buffer according to standard procedures. Pleural fluid samples were obtained from congestive heart failure patients and determined to be transudative based on a pleural fluid LDH to serum LDH ratio of < 0.60. Non-malignant ascites samples were obtained from liver disease patients and were also found to be transudates. Aliquots of serum, pleural fluid, and ascites were heated to 57°C for 30 minutes to produce heat-inactivated serum, pleural fluid, and ascites respectively.

NK cell activation

NK cell activation was measured using a phenotypic assay. This assay uses fresh normal donor PBMCs as a source of NK cells and Raji cells as CD20-positive target cells (91). PBMCs and Raji cells were mixed at a 1:1 effector-target (E/T) ratio at a final concentration of 1 X 10^6 PBMCs and 1 X 10^6 Raji cells/mL. Various concentrations or rituximab, serum, ascites or pleural fluid (untreated, heat-inactivated or treated with CVF or HC3-1496) were added as indicated. Samples were then cultured for 20 hours at 37°C prior to phenotypic analysis.

NK cell phenotypic analysis

Immunofluorescent staining was performed on co-cultured cells to assess changes in NK cell phenotype. Briefly, cells were washed and stained with directly conjugated commercial antibodies, including anti-human CD56 AlexaFluor 647, CD54 PE (BD Pharmingen), CD16 FITC (Serotec, Raleigh, NC), and CD3 PE-Cy7 (Caltag Laboratories, Burlingame, CA) per the manufacturer's protocol for 15 minutes on ice. Cells were washed twice, fixed in 2% formaldehyde solution, and stored at 4°C for flow cytometry within 24 hours. Flow cytometric analysis (4-color) was performed on the LSR II (BD Immunocytometric Systems, San Jose, CA). Data were analyzed using FlowJo software (TreeStar, Ashland, OR). CD16 and CD54 expression of NK-cells was determined by gating on $CD3^-$, $CD56^+$ lymphocytes, CD16 expression was reported as median fluorescence. In prior studies, we found that NK-cell expression of CD54 was bimodal, with activated NK cells expression bright CD54. Therefore, NK-cell activation is reported as the percentage of NK cells that were CD54 bright.

38C13 murine lymphoma model

The 38C13 murine lymphoma tumor model was used for in vivo experiments (103, 175, 178, 179). Female C3H mice were purchased from Harlan Biosciences (Indianapolis, IN) and housed in the University of Iowa Animal Care Facility. 5 X 10^3

38C13 cells growing in log phase were injected i.p. into 6-9 week old mice. Day 0 was defined as the day of tumor inoculation. Mice were injected with 10μg of CVF or HC3-1496 in 0.1 ml of PBS on Day 3 and Day 5. Mice were treated with doses of 0.1 mg of MS11G6 anti-lymphoma mAb in 0.2 ml PBS i.p. 4 hours after the initial CVF or HC3-1496 injection. The University of Iowa animal care and use committees approved all of the mice experiments.

Results

Complement activity is present in extravascular fluid

It was previously demonstrated that complement in serum inhibits NK cell activation induced by rituximab-coated target cells (174). However, the majority of malignant cells in most lymphoma patients are in the lymph nodes, an extravascular compartment. This finding would be of potential clinical relevance if extravascular fluid contained enough complement to interact with rituximab-coated target cells. Remarkably little is known about presence of complement in the extravascular compartment. I therefore assessed for complement activity in extravascular fluid by using transudative pleural fluid and ascites from non-malignant patients as surrogates. An ELISA demonstrated that C1q is present in serum, pleural fluid, and ascites with concentrations of C1q higher in serum than in the surrogates for extravascular fluid (Figure 15). However, the complement in pleural fluid and ascites was sufficient to induce CDC of rituximab-coated Raji cells. Pleural fluid induced lysis of target cells at concentrations of rituximab above 1 μg/ml while ascites induced lysis of target cells at the rituximab concentration of 5μg/ml (Figure 16). These results suggest that complement is present in pleural fluid and ascites at concentrations high enough to mediate complement activity. In addition, it has been shown that C3 is deposited on rituximab-coated Raji cells when incubated with pleural fluid or ascites at levels above 3.125% (180).

Complement activity in extravascular fluid inhibits NK activation

To determine whether complement activity in extravascular fluid inhibits NK cell activation, PBMCs from healthy donors were cultured with Raji cells and rituximab in the presence of pleural fluid or ascites. With results similar to serum, pleural fluid and ascites inhibited the down-modulation of NK cell CD16 induced by rituximab-coated targets at rituximab concentrations greater than 0.04 μg/ml (Figure 17A). Both pleural fluid and ascites also inhibited the upregulation of NK cell CD54 at rituximab concentrations greater than 0.04 μg/ml (Figure 17B). Similar results were seen when CD69 and IFN-γ was used as markers of NK cell activation (data not shown). Together, these data suggest that complement activity in pleural fluid and ascites both inhibit NK cell activation induced by rituximab-coated target cells.

The inhibitory effects of extravascular fluid are dose-dependent and abrogated with heat inactivation

To investigate whether the effects of extravascular fluid were dose dependent, the amount of pleural fluid and ascites was titrated in vitro. The inhibitory effects on NK cell CD16 down-modulation were greater as the concentration of pleural and ascites fluid was increased (Figure 18A). Similarly, the inhibition of NK cell CD54 was also dependent on the concentration of extravascular fluid, with greater inhibition at higher concentrations of pleural fluid or ascites (Figure 18B). Much like serum, the inhibitory effects of pleural fluid or ascites were abrogated by heat inactivation, suggesting that the inhibition is due to complement activity (Figures 18A and 18B).

Depletion of complement enhances NK cell activation

CVF is a structural and functional analog of the complement component, C3, which forms a bimolecular enzyme able to proteolytically activate C3 and C5. In contrast to the C3b-containing enzyme, the CVF-containing enzyme is stable and resistant to

control mechanisms, resulting in depletion of complement activity (181). In order to determine whether depletion of complement activity enhances NK cell activation, pleural fluid and ascites were incubated with CVF prior to use in the assay. In the absence of CVF treatment, NK cell CD54 upregulation was inhibited by both the pleural fluid and ascites as described above. When serum, pleural fluid, and ascites were incubated with CVF, the inhibitory effect was abrogated, resulting in increased NK cell CD54 upregulation at rituximab concentrations greater than 0.04 μg/ml (Figures 19). These results suggest that depletion of C3 enhances NK cell activation in the presence of complement and provide further evidence that the observed inhibitory effect is due to complement activity.

Murine serum blocks mAb-induced activation of murine NK cells

Previous studies have demonstrated that the in vivo therapeutic activity of murine anti-idiotype mAb (MS11G6) against the murine 38C13 B-cell lymphoma cell line is dependent, at least in part, on NK cells (179). This model also has the advantage of utilizing an anti-lymphoma mAb in syngeneic immunocompetent mice. Initial studies have also demonstrated that 38C13 murine lymphoma cells coated with MS11G6 induce activation of murine NK cells as indicated by upregulation of CD69 and production of IFN-γ (180). In contrast to the human system, in which NK cell CD16 was down-modulated by mAb-coated targets, there was no change in murine NK cell CD16/32. These differences are likely due to the inherent differences between human and murine systems. There were no changes in murine NK phenotype when 38C13 cells lacking the idiotype target antigen were used as targets (data not shown).

In addition, syngeneic murine serum was found to inhibit the activation of murine NK cells by mAb-coated murine targets. This inhibition was abrogated when the murine serum was heat-inactivated (180).

Depletion of complement improves the efficacy of monoclonal antibody therapy in a murine B cell lymphoma model

I next used the 38C13 model to evaluate the effects of depleting complement activity in an in vivo system. In addition to CVF, a human C3 derivative protein with CVF-like functions (HC3-1496) was used to deplete C3 in C3H/HeN mice. HC3-1496 resembles CVF in its C3-cleaving activity, but does not activate C5. In vivo administration of both CVF and HC3-1496 has been shown to safely deplete complement in murine models (181, 182).

To assess whether depletion of complement in vivo impacts NK cell activation induced by rituximab-coated targets, serum from mice treated with CVF or HC3-1496 were added to the co-culture assay. Serum from untreated mice inhibited NK cell CD54 upregulation induced by rituximab-coated target cells. The inhibitory effect was significantly decreased in serum from mice treated with CVF or HC3-1496 (Figure 20).

I next used the 38C13 model to determine whether depletion of complement impacts on the efficacy of an anti-lymphoma mAb therapy. Mice were injected with 38C13 lymphoma cells and treated with a single dose of MS11G6 on day 3 after the tumor inoculation. To deplete complement, mice were treated with CVF or HC3-1496 four hours prior to MS11G6 treatment and once again on day 5. Untreated mice all developed tumor and died with a median survival of 28 days. MS11G6 treatment alone increased the median survival to 42 days. Treatment with CVF enhanced the efficacy of the anti-lymphoma mAb therapy with 50 percent of mice remaining tumor free ($p=0.0312$) (Figure 21A). HC3-1496 treatment also enhanced the efficacy of MS11G6 with 80 percent of mice remaining tumor free ($p=0.0002$) (Figure 21B). These data suggest that complement depletion by CVF or HC3-1496 improves the efficacy of a mAb therapy in a murine B cell lymphoma model.

Discussion

The role of complement in mediating the anti-tumor activity of mAbs has been controversial. Various studies have demonstrated that complement can induce in vitro lysis of mAb-coated target cells (17, 27-29). However, I reported in Chapter II that complement can also inhibit the killing of target cells by blocking NK cell-mediated ADCC(174). Studies investigating the clinical significance of these in vitro findings are necessary to determine whether complement is indeed "friend or foe" to the therapeutic response of rituximab in B cell malignancies.

Normal human serum has been traditionally utilized to study mechanisms involving complement. However, the majority of malignant lymphocytes reside in the extravascular compartment, where protein concentrations differ from those in circulation. The lack of appropriate methods of obtaining extravascular fluid has prevented the direct evaluation of complement activity in extravascular compartments. Both transudative pleural fluid and ascites from patients with non-malignant disease are filtered through the normal endothelial barrier in a manner similar to extravascular fluid. I therefore used transudative pleural fluid from congestive heart failure patients and ascites from non-malignant liver disease patients as surrogates to assess how complement in the extravascular fluid impacts on the anti-tumor effects of mAbs.

Both congestive heart failure and cirrhosis has been known to induce systemic inflammation, resulting in complement protein consumption (183, 184). Thus, pleural fluid and ascites are not ideal surrogates for extravascular fluid. The concentration of active complement in these surrogates may be less than what is expected to be found in the extravascular compartments. However, both transudative pleural fluid and ascites displayed significant complement activity. Complement in both pleural fluid and ascites mediated CDC of rituximab-coated target cells and inhibited NK cell activation induced by rituximab-coated targets. These findings suggests that the ability of complement to

induce lysis of mAb-coated target cells and the inhibit NK cell activation may be present in lymph nodes and other sites outside the vascular compartment.

Since NK cell-mediated ADCC seems to play an important role in the therapeutic effects of mAbs, depletion of the inhibitory effects of complement would likely enhance the efficacy of therapy. C3 was shown in Chapter II to be necessary for the inhibitory effect of serum on NK cell activation(174). Because CVF is a reagent commonly used in studies to deplete complement activity, the use of CVF or a humanized protein with CVF-like activity to deplete C3 theoretically provides a targeted approach to enhance ADCC.

The in vitro results of the NK cell activation studies are consistent with this hypothesis. Incubation of serum, pleural fluid, and ascites with CVF reversed the ability of complement to block NK cell activation induced by rituximab-coated target cells. Inhibition of NK activation by mouse serum was also abrogated when mice were treated with CVF in vivo.

Correlative, yet convincing studies suggest that NK cell CD16 is an important mediator of the clinical response of rituximab (80-82). In vivo testing of ADCC requires a model where NK cells are known to contribute to the effects of therapy. The anti-tumor effect of the anti-idiotype, MS11G6, in mice inoculated with 38C13 has been shown to be dependent, at least in part, on NK cells (179). The target antigen in this model is different from that of rituximab, however it is a syngeneic murine lymphoma system previously used to study ADCC (103, 175, 178, 179, 185, 186). I therefore used the 38C13 model to assess the effect of complement depletion on the efficacy of mAb therapy.

While CVF has been widely used in animal models to deplete complement, it is highly immunogenic, which limits its potential clinical application. To address this issue, a hybrid protein was engineered in which a portion of the alpha-chain at the C-terminus of human C3 is replaced with homologous regions of the CVF beta-chain crucial to

activity. Using both CVF and HC3-1496, it was found that depletion of complement in the 38C13 model improved the efficacy of mAb therapy.

Further studies are needed to evaluate whether complement activity inhibits mAb-induced NK cell activation in patients. If complement indeed limits the clinical response of mAbs, then the use of agents such as HC3-1496 to deplete complement prior to mAb therapy may enhance therapy. Alternative strategies to addressing the effect of complement include the use of complement inhibitors (as opposed to enzymatically depleting complement with CVF and HC3-1496) or the use of modified anti-tumor mAbs lacking complement fixing capabilities. Each of these approaches is currently under evaluation, as is the assessment of the relationship between complement and NK cell activation in patients undergoing rituximab treatment.

In summary, it was previously reported that complement in serum can limit the ability of mAb-coated targets to activate NK cells. The results in this chapter suggest that the complement present in extravascular fluid has similar effects. In addition, the ability of mAb-coated targets to activate NK cells in the presence of serum or extravascular fluid is enhanced by complement depletion. Most importantly, in vivo depletion of complement enhanced the efficacy of an anti-lymphoma mAb therapy in a syngeneic mouse model. These studies provide further evidence that complement fixation can impede the anti-tumor effects of mAbs. They also suggest that reversing the inhibitory effects of complement may be a potential approach to enhance NK cell-mediated lysis of malignant cells and improve the efficacy of mAbs. Clinical studies will need to be performed before we can fully understand how to take advantage of the complex interactions between complement and effector cell-mediated mechanisms of mAb therapy.

Figure 15. C1q is present in transudative pleural fluid and non-malignant ascites fluid.

C1q protein was quantified in samples of serum, transudative pleural fluid and non-malignant ascites fluid using ELISA (n = 3 samples per group). C1q levels are reported in μg/ml. Error bars represent the standard deviation (SD) of the mean.

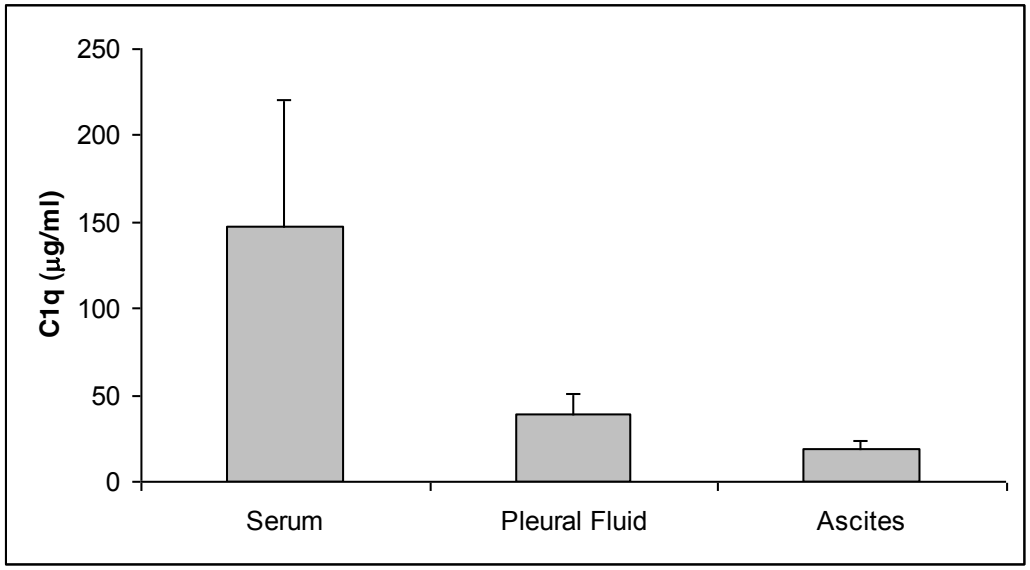
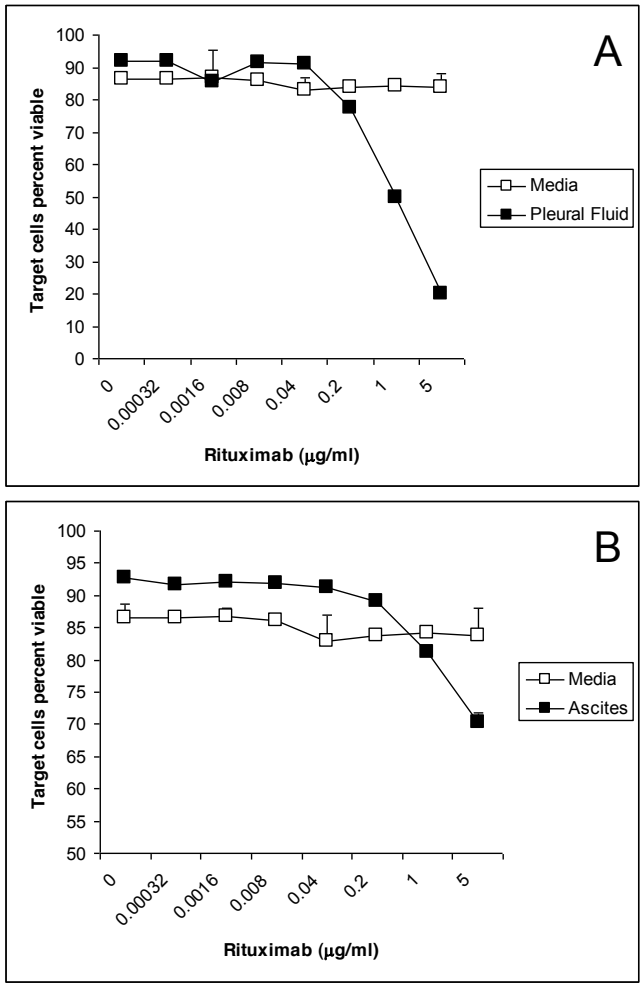

250
200
150
100
50
0
C1q (µg/ml)
Serum
Pleural Fluid
Ascites

Figure 16. Transudative pleural fluid and non-malignant ascites mediate complement activity.

Raji cells were incubated for 20 hours in media, 50% pleural fluid, or 50% ascites with various concentrations of rituximab. The percent of viable target cells were determined using flow cytometry by counting annexin V and propidium iodide-negative target cells (n=3). Error bars represent SD of the mean. Statistical difference ($P<0.05$) between media and pleural fluid was noted at rituximab concentrations greater than 0.2μg/ml by paired student t-test. Statistical difference ($P<0.05$) between media and ascites was noted at rituximab concentrations greater than 1μg/ml by paired student t-test.


A
Target cells percent viable
100
90
80
70
60
50
40
30
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10
0
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0.00032
0.0016
0.008
0.04
0.2
1
5
Rituximab (μg/ml)
Media
Pleural Fluid
B
Target cells percent viable
100
95
90
85
80
75
70
65
60
55
50
0
0.00032
0.0016
0.008
0.04
0.2
1
5
Rituximab (μg/ml)
Media
Ascites

Figure 17. Transudative pleural fluid and non-malignant ascites inhibit rituximab induced NK-cell CD16 down-modulation and CD54 up-regulation.

PBMCs and Raji cells were mixed at a 1:1 ratio for 20 hours in the presence of absence of 50% pleural fluid or ascites with varying concentrations of rituximab. NK cell surface marker expression was determined by flow cytometry with gating on CD3-, CD56+ lymphocytes. (A) NK-cell CD16, expressed as median fluorescence, in the absence and presence of pleural fluid or ascites fluid (n = 3 samples per group). (B) NK-cell CD54, expressed as a percentage of CD54 bright, in the absence and presence of pleural fluid or ascites fluid (n = 3 samples per group). Error bars represent the SD of the mean. Statistical difference ($P<0.05$) between media and pleural fluid was noted at rituximab concentrations greater than 0.04μg/ml by paired student t-test. Statistical difference ($P<0.05$) between media and ascites was noted at rituximab concentrations greater than 0.04μg/ml by paired student t-test.

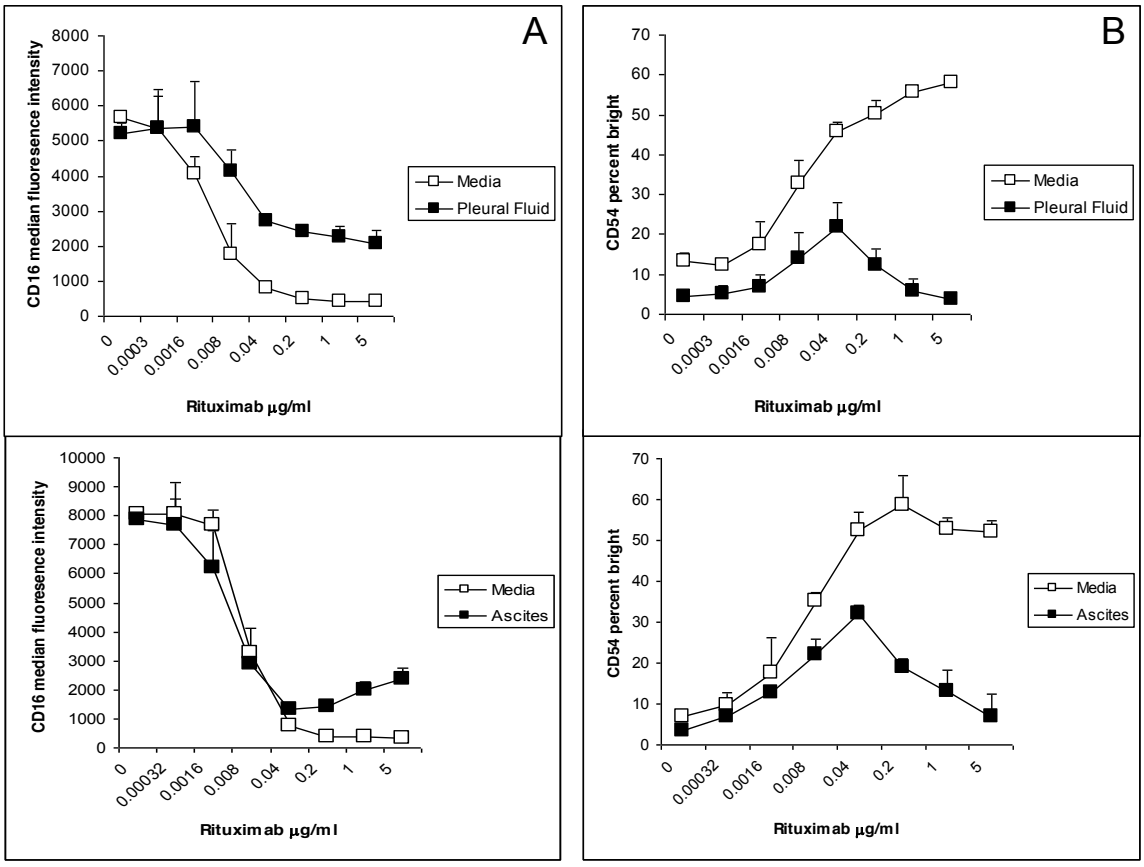

A
CD16 median fluoresence intensity
8000
7000
6000
5000
4000
3000
2000
1000
0
0
0.0003
0.0016
0.008
0.04
0.2
1
5
Rituximab µg/ml
Media
Pleural Fluid
B
CD54 percent bright
70
60
50
40
30
20
10
0
0
0.0003
0.0016
0.008
0.04
0.2
1
5
Rituximab µg/ml
Media
Pleural Fluid
CD16 median fluoresence intensity
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
0.00032
0.0016
0.008
0.04
0.2
1
5
Rituximab µg/ml
Media
Ascites
CD54 percent bright
70
60
50
40
30
20
10
0
0
0.00032
0.0016
0.008
0.04
0.2
1
5
Rituximab µg/ml
Media
Ascites

Figure 18. Inhibitory effect of transudative pleural fluid and non-malignant ascites is dose dependent and abrogated by heat inactivation.

PBMCs and Raji cells were mixed at a 1:1 ratio for 20 hours in varying concentrations of pleural fluid, ascites, or heat-inactivated samples in the presence of 5 μg/ml rituximab. NK cell surface marker expression was determined using flow cytometry with gating on CD3-, CD56+ lymphocytes. (A) NK-cell CD16, expressed as median fluorescence, after incubation with varying concentrations of pleural fluid, ascites, or heat-inactivated samples (n = 3 samples per group). (B) NK-cell CD54, expressed as a percentage of CD54 bright, after incubation with varying concentrations of pleural fluid, ascites, or heat-inactivated samples (n = 3 samples per group). Error bars represent SD of the mean. Statistical difference ($P<0.05$) between pleural fluid and heat-inactivated pleural fluid was noted at concentrations greater than 12.5% by paired student t-test. Statistical difference ($P<0.05$) between ascites and heat-inactivated ascites was noted at concentrations greater than 25% by paired student t-test.

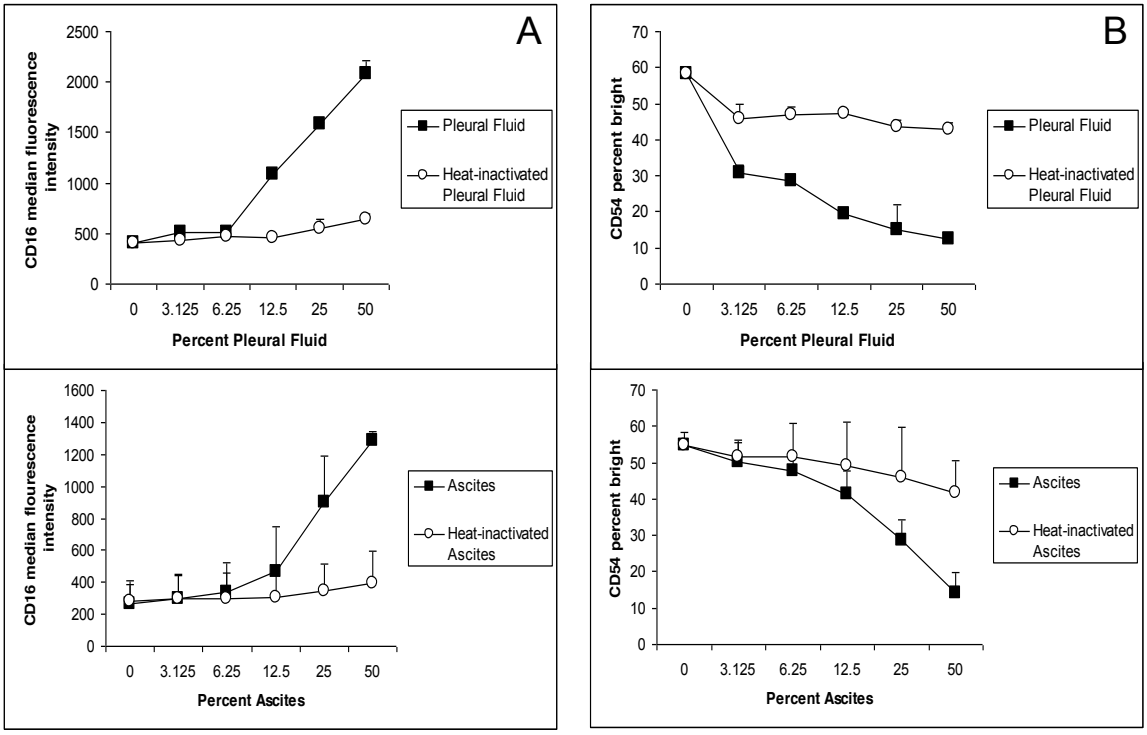
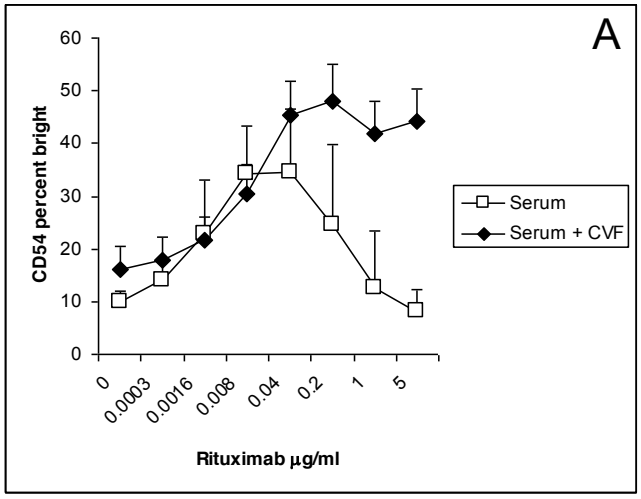

A
CD16 median fluorescence intensity
2500
2000
1500
1000
500
0
0
3.125
6.25
12.5
25
50
Percent Pleural Fluid
Pleural Fluid
Heat-inactivated Pleural Fluid
B
CD54 percent bright
70
60
50
40
30
20
10
0
0
3.125
6.25
12.5
25
50
Percent Pleural Fluid
Pleural Fluid
Heat-inactivated Pleural Fluid
CD16 median flourescence intensity
1600
1400
1200
1000
800
600
400
200
0
0
3.125
6.25
12.5
25
50
Percent Ascites
Ascites
Heat-inactivated Ascites
CD54 percent bright
70
60
50
40
30
20
10
0
0
3.125
6.25
12.5
25
50
Percent Ascites
Ascites
Heat-inactivated Ascites

Figure 19. Cobra venom factor (CVF) enhances NK-cell CD54 upregulation in the presence of complement.

PBMCs and Raji cells were mixed at a 1:1 ratio for 20 hours in the presence of 50% serum, pleural fluid, or ascites with or without the addition of CVF in varying concentrations of rituximab. NK cell surface marker expression was determined using flow cytometry with gating on CD3-, CD56+ lymphocytes. (A) NK-cell CD54, expressed as a percentage of CD54 bright, after culture in serum or serum plus CVF (n = 3 samples per group). Statistical difference ($P<0.05$) between serum and serum + CVF was noted at rituximab concentrations greater than 0.2μg/ml by paired student t-test. (B) NK-cell CD54, expressed as a percentage of CD54 bright, after culture in pleural fluid or pleural fluid plus CVF (n = 3 samples per group). Statistical difference ($P<0.05$) between pleural fluid and pleural fluid + CVF was noted at rituximab concentrations greater than 0.2μg/ml by paired student t-test. (C) NK-cell CD54, expressed as a percentage of CD54 bright, after culture in ascites or ascites plus CVF (n = 3 samples per group). Statistical difference ($P<0.05$) between ascites and ascites + CVF was noted at rituximab concentrations greater than 0.2μg/ml by paired student t-test. Error bars represent SD of the mean.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CD54 percent bright
60
50
40
30
20
10
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0
0.0003
0.0016
0.008
0.04
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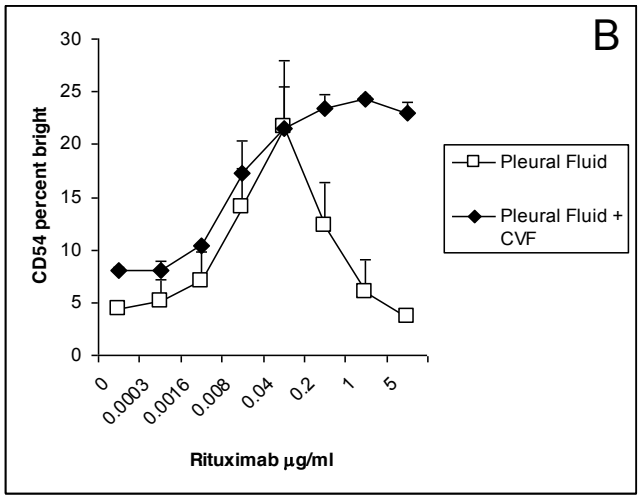
1
5
Rituximab µg/ml
Serum
Serum + CVF


B
CD54 percent bright
30
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0.0003
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0.04
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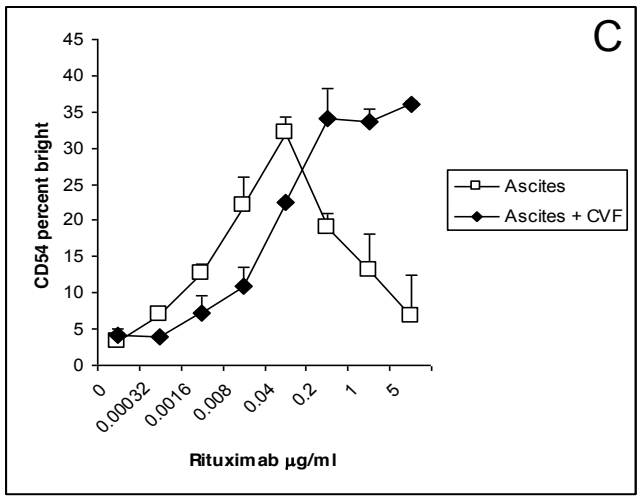
1
5
Rituximab µg/ml
Pleural Fluid
Pleural Fluid + CVF


C
CD54 percent bright
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40
35
30
25
20
15
10
5
0
0
0.00032
0.0016
0.008
0.04
0.2
1
5
Rituximab µg/ml
Ascites
Ascites + CVF

Figure 20. Inhibitory effect of murine serum is abrogated by CVF or HC3-1496 injection.

PBMCs and Raji cells were mixed at a 1:1 ratio for 20 hours in the presence of 50% serum from untreated mice, mice injected with CVF, or mice injected with HC3-1496 in 5 μg/ml of rituximab. NK cell surface marker expression was determined using flow cytometry with gating on CD3-, CD56+ lymphocytes. NK-cell CD54, expressed as a percentage of CD54 bright, was determined (n = 3 mice per group). Error bars represent SD of the mean. Statistical difference (P=0.0001) between serum and serum + CVF was noted by paired student t-test. Statistical difference (P=0.0001) between serum and serum + HC3-1496 was noted by paired student t-test.

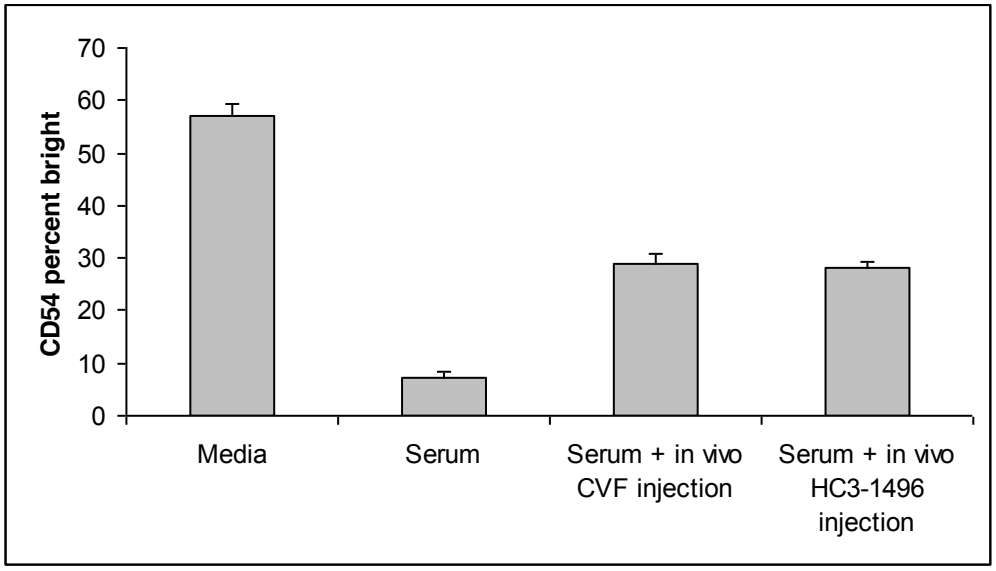
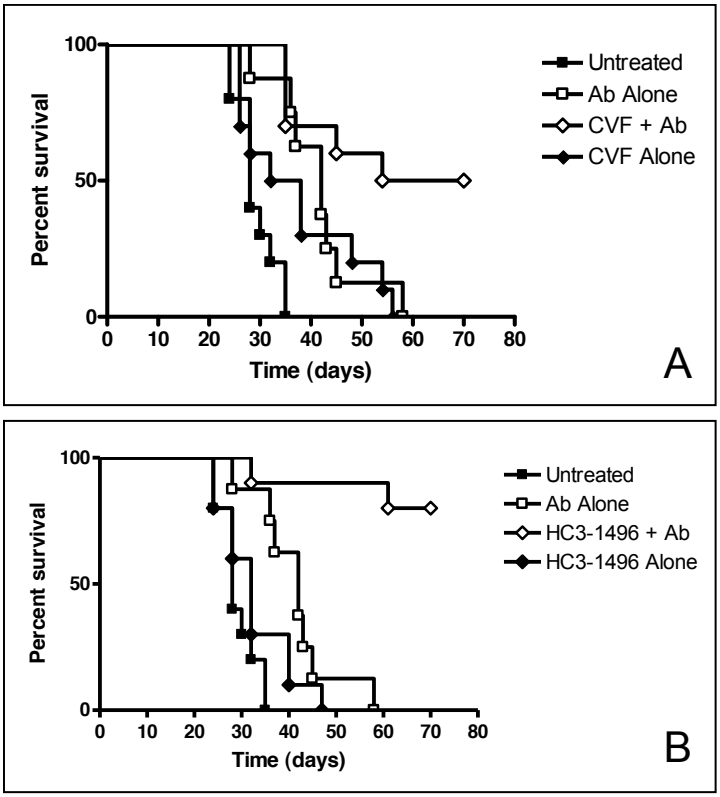

CD54 percent bright
70
60
50
40
30
20
10
0
Media
Serum
Serum + in vivo CVF injection
Serum + in vivo HC3-1496 injection

Figure 21. CVF and HC3-1496 enhance the efficacy of monoclonal antibody therapy in vivo.

Mice were inoculated i.p. with 38C13 tumor cells on day 0. Select groups of mice were treated with anti-lymphoma IgG2a (MS11G6) on day 3, CVF on day 3 and day 5, or HC3-1496 day 3 or day 5. (A) Survival of mice treated with MS11G6 alone, MS11G6 plus CVF, CVF alone, or untreated. Statistical difference (P=0.0312) between MS11G6 and MS11G6 + CVF was noted by the log-rank test. (B) Survival of mice treated with MS11G6 alone, MS11G6 plus HC3-1496, HC3-1496 alone, or untreated. Statistical difference (P=0.0002) between MS11G6 and MS11G6 + HC3-1496 was noted by the log-rank test. (n = 10 mice per group)


100
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Percent survival
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Time (days)
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CVF Alone
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Time (days)
Untreated
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HC3-1496 + Ab
HC3-1496 Alone
B

CHAPTER IV

NK CELL ACTIVATION CORRELATES WITH DECREASED COMPLEMENT ACTIVITY IN PATIENTS FOLLOWING RITUXIMAB TREATMENT

Abstract

NK cell mediated antibody-dependent cellular cytotoxicity is thought to play a role in the anti-tumor effects of rituximab. Recent studies have suggested that complement activity inhibits NK cell activation and function. This study was designed to evaluate the relationship between NK cell activation and complement activity in patients after rituximab treatment. Changes in the phenotypic markers of NK cell activation, CD16 and CD54, correlated with a decrease in complement activity. Serum levels of NK cell-secreted cytokines, IFN-γ and TNF-α, also correlated with a decrease in complement activity. These findings suggest that NK cell activation occurs when complement activity is decreased. Further studies are needed to determine whether complement inhibits NK cell activation in patients following rituximab treatment.

Introduction

Rituximab, a chimeric anti-CD20 monoclonal antibody (mAb), was the first mAb to be used in cancer therapy. Currently, it is used alone or in combination with other agents to treat various B-cell non-hodgkin's lymphomas (NHL). There is a 48% overall response rate in follicular lymphoma patients treated with rituximab with a median time to progression of 13 months (11, 187). After relapse, patients treated with rituximab have similar response rates with a possible extended period of remission (12, 188). In addition, rituximab is now part of the standard treatment for diffuse large B-cell lymphoma and has

been shown to be effective in marginal zone lymphoma, mantle cell lymphoma, and chronic lymphocytic leukemia (189).

Even though rituximab is widely used in the clinics, the mechanisms responsible for its therapeutic responses are still unclear. The binding of rituximab to CD20 has been shown to induce apoptosis of certain B cell lines, but the importance of signaling-induced apoptosis has yet to be demonstrated clinically (121). Various studies suggest that antibody-dependent cellular cytotoxicity (ADCC) is important for the therapeutic effect of mAbs. Clynes et al demonstrated that Fc receptors are crucial for the anti-tumor activity of mAbs in several tumor models (76, 77). More convincingly, patients homozygous for the V158 (VV) polymorphism on CD16 demonstrate higher affinities to human IgG and have better clinical response rates to rituximab than carriers for F158 (VF or FF) (80-82). These data suggest that ADCC plays a significant role in the anti-tumor effects of rituximab.

As with ADCC, various studies suggest that complement-dependent cytotoxicity (CDC) contributes to the therapeutic effect of rituximab (36, 38, 146). However, complement fixation and lymphoma cell expression of complement inhibitory proteins, CD55 and CD59, do not correlate with clinical response to therapy (29). In addition, several studies have now suggested that complement activation may actually be inhibiting the therapeutic effect of mAbs. The previous chapters show that complement activation blocks the interaction between the Fc portion of rituximab and NK cell CD16. This interaction inhibits the activation of NK cells and ADCC (174). It was also found that follicular lymphoma patients with a polymorphism in C1qA associated with lower C1q levels (AA) correlates with a prolonged response to rituximab (132). In addition, Cragg and Glennie have shown that type II mAbs, which demonstrate less CDC, are more effective than the complement activating type I mAbs in depleting B-cells in vivo (190). However, it is not clear whether the differences observed between type I and type II mAbs are due to complement.

The recent finding that complement inhibits NK cell activation suggests that complement activity may be limiting the efficacy of rituximab. To further investigate the relationship between complement and ADCC, I examined NK cell surface phenotypes, NK cell-secreted cytokines, and complement activity in patients after rituximab treatment. It was found that after 4 hours of treatment, the complement activity of serum was decreased in a subset of patients. The decrease in complement activity correlated with evidence of NK cell activation, indicated by NK cell CD16 down-modulation, NK cell CD54 upregulation, serum IFN-γ, and serum TNF-α levels. These findings suggest that NK cell activation occurs when complement activity is decreased and support the hypothesis that complement activation inhibits NK cell mediated ADCC.

Materials and methods

Patients and samples

Subjects include NHL patients receiving their first dose of standard rituximab therapy (375 mg/M^2 weekly X 4) using standard infusion guidelines. The patients have not received rituximab treatment in the previous six months. Peripheral blood and serum samples were collected prior to and 4 hours after initiation of rituximab. Complete blood counts of patients were also obtained at those time points. The study was conducted according to protocols approved by the institutional review boards at the University of Iowa. Informed consent was obtained from all patients before sample collection.

Determination of rituximab concentration

The concentration of rituximab in patient serum was determined by sandwich ELISA. Briefly, the ELISA plates are coated overnight with 1μg/ml of rat anti-rituximab idiotype (AbD Serotec, Raleigh, NC). After washing and blocking, patient serum and standards are added in binary dilution and incubated for two hours at room temperature. The plates are then washed five times with PBS-T and incubated with HRP-conjugated

goat or rabbit anti-human Ig detection antibody for one hour at room temperature. After final wash, the ELISA is developed in the presence of TMB substrate and stopped with 0.6N H_2SO_4. The absorbance is measured at 450nm with background subtracted at 650nm.

NK cell phenotypic analysis

Peripheral blood mononuclear cells (PBMCs) were isolated and red blood cells were removed by resuspending cells in 5 mL red cell lysis buffer according to standard procedures. Immunofluorescent staining was performed on PBMCs (91, 151). Briefly, cells were washed and stained with directly conjugated commercial antibodies, including anti-human CD56 AlexaFluor 647, CD54 PE (BD Pharmingen), CD16 FITC (Serotec, Raleigh, NC), and CD3 PE-Cy7 (Caltag Laboratories, Burlingame, CA) per the manufacturer's protocol for 15 minutes on ice. Cells were washed twice, fixed in 2% formaldehyde solution, and stored at 4°C for flow cytometry within 24 hours. Flow cytometric analysis (4-color) was performed on the LSR II (BD Immunocytometric Systems, San Jose, CA). Data were analyzed using FlowJo software (TreeStar, Ashland, OR). CD16 and CD54 expression of NK cells was determined by gating on $CD3^-$, $CD56^+$ lymphocytes, CD16 expression was reported as median fluorescence. In prior studies, we found that NK-cell expression of CD54 was bimodal, with activated NK cells expressing bright CD54. Therefore, NK-cell activation is reported as the percentage of NK cells that were CD54 bright.

Serum cytokine analysis

For the simultaneous measurement of multiple cytokines in serum samples, a LINCO*plex* kit was used according to manufacturer's protocol (Linco research, St. Charles, MO). A filter-bottom, 96-well microplate was pre-wet for 10 minutes with provided assay buffer. A standard curve (ranging from 0 to 2000 pg/mL) was made by 5-fold dilutions of the human cytokine standard cocktail in the provided buffer. Standards,

controls, and serum samples were pipetted at 50 μL per well in duplicate. 50 μL of provided assay buffer was added to the serum samples. 50 μL of serum matrix solution was added to each well. After adding 25 μL of the bead mixture, the microplate was incubated overnight on a microtiter shaker in the dark at room temperature. Wells were washed twice using a vacuum manifold. 50 μL of provided detection antibody cocktail was added, and the microplate was incubated for 1 hour in the dark on a microtiter shaker. After 30 min of incubation with streptavidin-phytoerythrin, the microplate was washed twice. Sheath fluid was added to each well, and samples were analyzed using the Bioplex system (Bio-Rad Laboratories, Hercules, CA).

CH50 Assay

For the determination of complement activity in serum samples, the EZ Complement CH50 Test (Diamedix, Miami, Fl) was used according to manufacturer's instructions. Briefly, 5 μl of patient samples, reference, high, or low controls were added to tubes of sensitized sheep erythrocytes and vortexed vigorously for 10 seconds. After incubation for 60 minutes, tubes were inverted 3-4 times and centrifuged at 1800 RPM for 10 minutes. Absorbances of the supernatants were read at 415 nm. CH50 values were determined by the following formula: CH50 value of sample = Absorbance of sample / Absorbance of reference X CH50 value of reference.

Results

Patient characteristics

The demographics and characteristics of the patients in this study are summarized in Table 2. Five subjects were enrolled in this study. The median age of the patients was 64 years. Subjects included one female and four male patients. Diagnoses of the patients included mantle cell lymphoma (MCL) with chronic lymphocytic leukemia (CLL), follicular lymphoma (FL), diffuse large B cell lymphoma (DLBCL), and B cell

lymphoproliferative process. Two of the patients received single agent rituximab while three patients received rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP). Two patients have had previous rituximab treatment.

Serum rituximab levels display high individual variability

An ELISA was performed to measure the concentration of rituximab in samples from patients 4 hours after the initiation of therapy. Serum rituximab concentrations are summarized in Table 3. There was great variability in patient serum rituximab concentrations, ranging from 83 μg/mL to 640 μg/mL.

Absolute lymphocyte counts decrease after rituximab infusion

Complete blood counts were obtained prior to and 4 hours after the initiation of therapy. The absolute lymphocyte counts are summarized in Table 3. The majority of patients showed a decrease in absolute lymphocyte counts after rituximab infusion. The greatest decrease was observed in patient 2, showing a relative change of 0.16 after therapy.

Flow-cytometry was performed on patient blood samples to determine the percentage of NK cells in the lymphocyte population. The NK cell counts are summarized in Table 3. There was variability in the NK cell counts after rituximab treatment. NK cell counts were decreased in three of the patients and elevated in two of the patients. The greatest decrease was in patient 1, showing a relative change of 0.24 after therapy. The greatest increase was observed in patient 4, demonstrating a relative change of 3.26 after therapy.

NK cell activation occurred in two patients after therapy

Previously, it was demonstrated that CD16 down-modulation and CD54 upregulation are appropriate markers of NK cell activation (91, 151). NK cell surface

phenotypes were analyzed using flow-cytometry on patient blood samples prior to and 4 hours after the initiation of therapy. Phenotypic surface markers on NK cells are summarized in Table 4. Significant NK CD16 down-modulation occurred in patients 1 and 2. The greatest amount of CD16 down-modulation occurred in patient 1, showing a relative change of 0.42 after therapy. NK cell CD54 upregulation was also demonstrated by patients 1 and 2. The greatest amount of CD54 upregulation also occurred in patient 1, showing a relative change of 7.77 after therapy.

To quantify the levels of NK cell-secreted cytokines in patient serum samples prior to and 4 hours after the initiation of therapy, a Bioplex system was used. Serum concentrations of IFN-γ and TNF-α are summarized in Table 4. Serum concentrations of IFN-γ were significantly higher in patients 1 and 2 after treatment. The greatest amount of IFN-γ elevation was observed in patient 2, demonstrating a relative change of 4.13 after treatment. Serum concentrations of TNF-α were also significantly elevated in patients 1, 2, and 5 after treatment. The greatest amount of TNF-α elevation was observed in patient 1, showing a 9.12 relative change after therapy.

Complement activity in serum samples was decreased in two patients after therapy

To assess complement activity in serum samples from patients prior to and 4 hours after the initiation of therapy, a CH50 assay was performed. CH50 values for each patient are summarized in Table 4. Complement activity in the serum was decreased in patients 1 and 2 after rituximab infusion. The most significant decrease was observed in patient 1, showing a 0.19 relative change after therapy. Serum samples from patient 2 also demonstrated a decrease in complement activity, showing a 0.64 relative change after therapy.

NK cell activation correlates with decreased complement activity

In order to evaluate the relationship between complement activity and NK cell activation, relative changes of CH50 values were compared to changes in NK cell surface markers and serum cytokine levels after initiation of therapy. Both NK cell CD16 down-modulation ($r = 0.9713$; $P = 0.0058$) (Figure 22A) and NK cell CD54 upregulation ($r = -0.9760$; $P = 0.0045$) (Figure 22B) correlated with the decrease in CH50 levels. Increases in serum concentrations of IFN-γ ($r = -0.78$; $P = 0.1192$) and TNF-α ($r = -0.8820$; $P = 0.0478$) also seemed to correlate with the decrease in CH50 levels (Figure 22C and 22D). However, the correlation of serum IFN-γ is not statistically significant. Altogether, these results suggest that NK cell activation correlates with decreased complement activity in patients following rituximab treatment.

Discussion

The role of complement in the therapeutic effects of rituximab remains controversial. There is evidence that complement is activated in patients following infusion of rituximab (40). However, whether complement contributes to the clinical response of rituximab is still unclear. In previous chapters, I demonstrated that complement activation inhibits NK cell-mediated ADCC. I've also shown that the depletion of complement enhances NK cell activation and improves a mAb therapy in a murine lymphoma model. These findings suggest that limiting complement activity may result in increased NK cell activation in patients following rituximab treatment. We assessed the relationship between complement and NK cell activation by analyzing peripheral blood and serum samples of patients prior to and 4 hours following therapy.

Even though all of the patients received a standard dose of rituximab, serum rituximab concentrations varied greatly following initiation of therapy. This variation is likely attributed to the diversity of the subjects. Patients included in this study had

different diagnoses and previous treatments. Differences in pharmacokinetics and tumor cell burden are also likely to influence serum rituximab concentrations in patients.

Absolute lymphocyte counts were found to be decreased in the majority of patients after rituximab infusion. The decrease is most likely due to the clearance of peripheral B cells induced by rituximab. In contrast, the NK cell counts varied among patients. Three patients displayed decreases in NK cell counts while two patients displayed increases. The decrease in the peripheral NK cell population could be the result of trafficking to extravascular compartments. It was previously shown that NK cells upregulate surface CD54 when activated by rituximab-coated targets (91, 151). This adhesion molecule is involved in interactions between endothelial cells and lymphocytes during diapedesis (191-195). Although not much is known about NK cell activity within lymph nodes, recent studies have suggested that NK cell recruitment to lymph nodes play a significant role in the adaptive immune system (196, 197). The observed regression of tumor-invaded lymph nodes after rituximab treatment also provides evidence for NK cell activity within the extravascular space. More studies are needed to investigate whether NK cells are recruited to the lymph nodes after activation by rituximab-coated targets.

Interestingly, patient 1 did not have the decrease in absolute lymphocyte count that the other patients demonstrated following rituximab treatment. Patient 1 also demonstrated the largest decrease in the peripheral NK cell population following treatment. This may be due to a high level of NK cell activation, resulting in trafficking of NK cells to the extravascular compartment before peripheral B cells are cleared in the vasculature. This hypothesis is supported by the fact that patient 1 showed higher levels of CD16 down-modulation, CD54 upregulation, and TNF-α production than the other patients. Continued enrollment of patients and analysis comparing the drop in peripheral NK cell population, lack of change in absolute lymphocyte count, and NK cell activation is needed to help determine if this explanation is true. Studies investigating the NK cell

population in the extravascular compartments and lymph nodes following rituximab infusion would directly test this hypothesis.

Another interesting finding was that patients that demonstrated significant NK cell activation (patients 1 and 2) had been previously been treated with rituximab (Table 1). The half-life of rituximab has been demonstrated to be 76.3 +/- 31.1 hours after the first infusion (198). None of the patients included in this trial have been exposed to rituximab in the prior six months and should be free from residual rituximab activity. However, it has been demonstrated that detectable antibody may be present up to six months after the end of treatment (198). Because a steady rise in preinfusion and postinfusion serum antibody levels occurs after each rituximab dose, it is possible that the previous exposure to rituximab in patients 1 and 2 resulted in the increase in NK cell activation. In addition, it has been reported that responders to rituximab therapy demonstrated significantly higher serum antibody levels than non-responders (198-200). However, patients 1 and 2 were measured to have a significantly lower concentration of serum rituximab (Table 3). Continued analysis of patient samples following additional infusions of rituximab would help determine the relationship between serum rituximab levels, NK cell activation, and complement activity.

Complement activity was found in the serum of all patients prior to rituximab infusion. Interestingly, two patients displayed significant decreases in complement activity following four hours of therapy. Various studies have demonstrated that rituximab is highly efficient in activating complement (17, 24-29). In addition, the complement activation products, C3b/c and C4b/c, can be measured in patients after rituximab treatment (40). It is possible that the continuous activation of complement consumes its components following the infusion of rituximab. This mechanism is effectively demonstrated by the complement depleting properties of cobra venom factor (CVF) (176). However, the level of consumption induced by rituximab likely depends on the concentration of complement proteins prior to treatment. Determining the serum

levels of complement proteins and complement activation products would test this hypothesis.

NK cell activation was found in two patients following infusion of rituximab. In addition, the activation of NK cells correlated with decreases in complement activity. These findings may be explained by the results described in previous chapters. It was previously shown that deposition of complement inhibits the interaction between NK cell CD16 and rituximab (174). Therefore, complement activity may be limiting NK cell activation during rituximab infusion. However, the presence of complement proteins on antibody-coated targets is dynamic and constantly removed by degradation factors. Once complement is consumed, proteins are cleaved without additional deposition. Theoretically, NK cells may interact with rituximab and become activated once complement activity is consumed. A summary of this hypothesis is depicted in Figure 23.

These preliminary studies demonstrate that NK cell activation is observed when complement activity is decreased. However, these findings are strictly correlative. Whether NK cell activation is the result or cause of decreased complement activity is unknown. Further investigations are needed to fully understand the relationship between decreased complement activity and NK cell activation. Studies assessing complement levels prior to rituximab infusion may determine whether complement activity inhibits NK cell activation after therapy. Following these patients long-term may also help determine the relative importance of complement and cellular-mediated mechanisms in the efficacy of mAb-based therapy.

Growing evidence suggest that effector cell-mediated mechanisms play a key role in the anti-tumor effects of rituximab. Recent findings suggest that complement activity may be inhibiting rituximab-induced NK cell activation and function. If true, then limiting complement activity may improve the efficacy of rituximab and other mAbs.

The use of CVF has been shown to effectively deplete complement activity in various systems. However, its immunogenicity makes it inappropriate for use in patients.

A human protein derivative with CVF-like functions (HC3-1496) has been engineered for clinical use. Trials involving the combination of rituximab with HC3-1496 may determine whether depletion of complement is an appropriate strategy to improve antibody therapy. In addition, many other complement inhibiting proteins are being evaluated for potential human use. These proteins include circulating complement regulators (C1 inhibitor, C4 binding protein, Factor H, Factor I, S protein, and clusterin) and soluble complement receptors (201). TP-10, a soluble complement receptor-1, has been shown to be a potent inhibitor of C3 and C5 convertase. Clinical trials have shown that TP-10 is safe and effective in inhibiting complement activation in patients following cardiopulmonary bypass surgery (202). It would be interesting to see if a combination of complement inhibitory proteins improve outcomes in lymphoma patients treated with rituximab.

In conclusion, this chapter demonstrated that NK cell activation after rituximab infusion occurs in patients displaying decreased complement activity. These findings support the hypothesis that NK cell activation occurs in the absence of complement. Further investigations are needed to determine whether complement inhibits NK cell activation after rituximab treatment and if depleting complement improves the efficacy of mAbs.

Table 2. Patient characteristics.

Patient	Age (years)	Gender	Diagnosis	Treatment	Previous rituximab
1	64	Male	MCL, CLL	Rituximab	Yes
2	72	Female	FL	Rituximab	Yes
3	59	Male	FL	R-CHOP	No
4	51	Male	DLBCL	R-CHOP	No
5	66	Male	B cell lymphoproliferative process	R-CHOP	No

Table 3. Absolute lymphocyte and NK cell counts.

Patient	Serum Rituximab (ug/ml)	Lymphocyte counts (K/mm^3)			NK cell Counts (cells/mm^3)		
		Pre	Post	Relative Change	Pre	Post	Relative Change
1	86	3440	3570	1.04	309	74	0.24
2	83	824	135	0.16	234	137	0.59
3	211	388	112	0.29	570	1170	2.053
4	272	533	212	0.4	752	2449	3.26
5	650	2640	590	0.22	377	160	0.42

Table 4. NK cell and complement activation

Patient	NK cell phenotypic markers					
	CD16 (Median Fluorescence Intensity)			CD54 (Percent Bright)		
	Pre	Post	Relative change	Pre	Post	Relative change
1	18957	7897	0.42	4.58	35.6	7.77
2	37269	29163	0.78	1.71	4.98	2.91
3	25824	28473	1.1	1.88	0.81	0.43
4	31793	31792	0.99	1.52	1.43	0.94
5	30826	29200	0.95	1.56	2.9	1.85
Patient	**Serum cytokine levels**					
	IFN-γ (pg/mL)			TNF-α (pg/mL)		
	Pre	Post	Relative change	Pre	Post	Relative change
1	8.19	28.46	3.47	24.24	221.03	9.12
2	4.05	16.71	4.13	64.15	559.57	8.71
3	14.16	16.17	1.14	3.56	7.74	2.17
4	0	0	1	5.25	7.92	1.51
5	6.86	11.99	1.75	6.85	27.8	4.06
Patient	**Complement activation**					
	CH50 value					
	Pre	Post	Relative change			
1	142.92	27.6	0.19			
2	131.04	84	0.64			
3	142.92	132.41	0.93			
4	115.03	115.81	1			
5	128.98	118.07	0.92			

Figure 22. Correlation between NK cell activation and complement activity.

(A) NK cell CD16 down-modulation correlated with the decrease in complement activity ($r = 0.9713$; $P = 0.0058$). (B) NK cell CD54 upregulation correlated with the decrease in complement activity ($r = -0.9760$; $P = 0.0045$). (C) IFN-γ secretion correlated with the decrease in complement activity. This correlation was not statistically significant ($r = -0.78$; $P = 0.1192$). (D) TNF-α correlated with the decrease in complement activity ($r = -0.8820$; $P = 0.0478$).

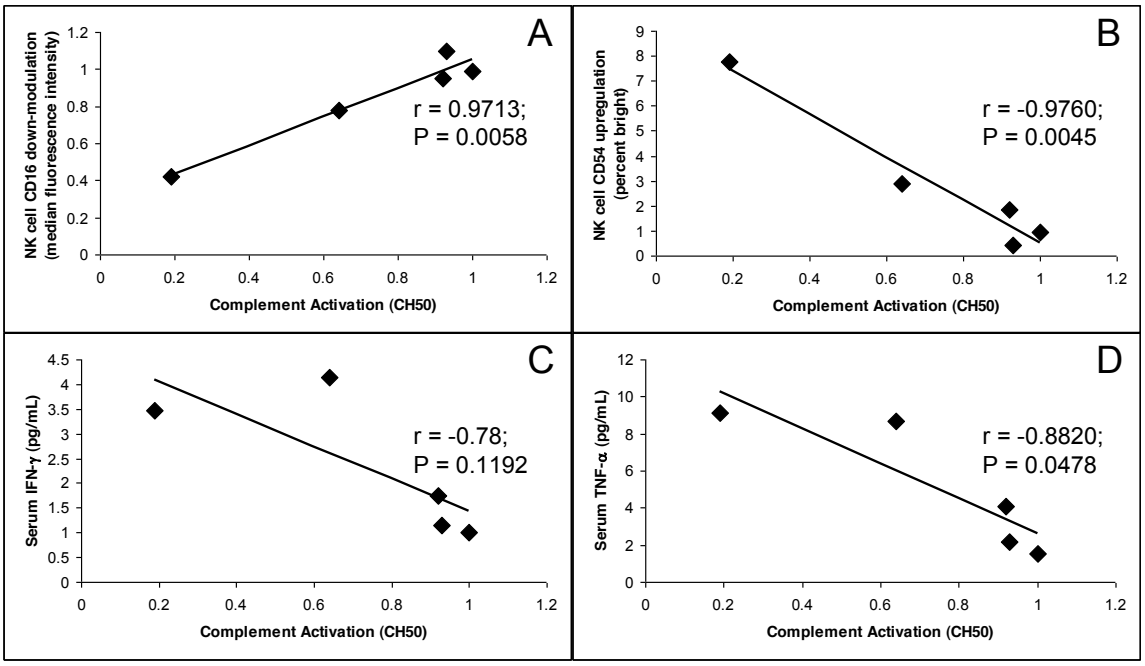
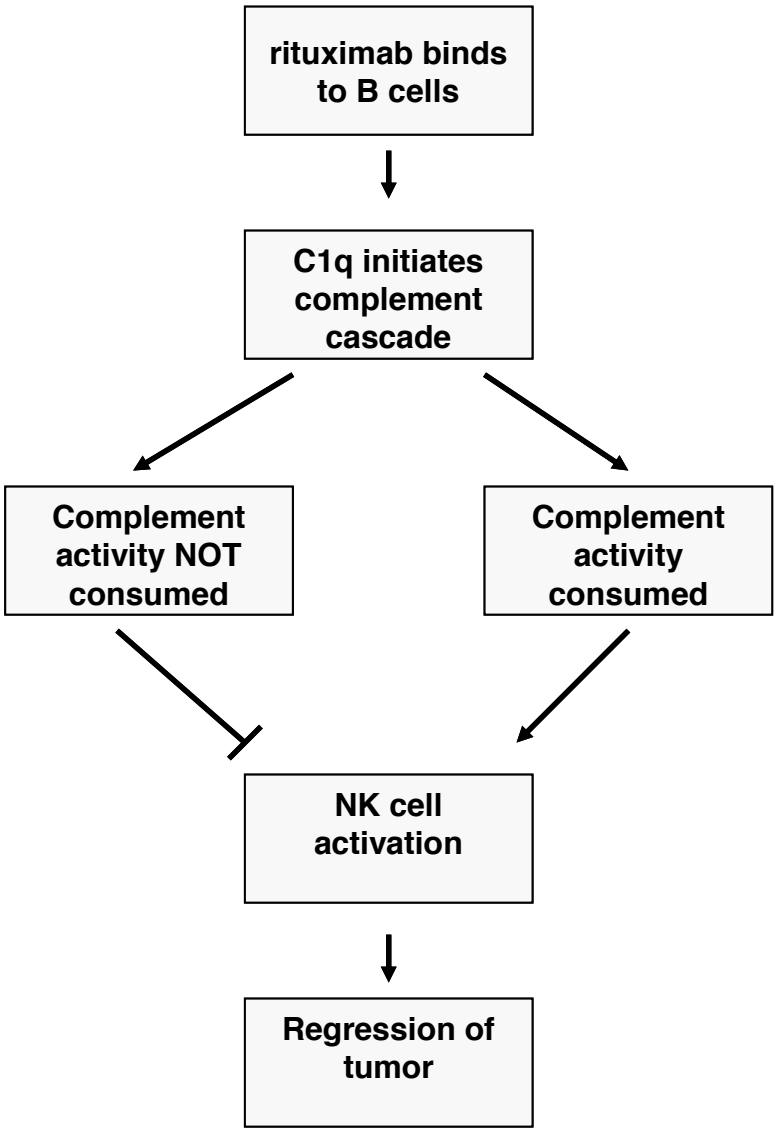

A
NK cell CD16 down-modulation (median fluorescence intensity)
r = 0.9713;
P = 0.0058
Complement Activation (CH50)
B
NK cell CD54 upregulation (percent bright)
r = -0.9760;
P = 0.0045
Complement Activation (CH50)
C
Serum IFN-γ (pg/mL)
r = -0.78;
P = 0.1192
Complement Activation (CH50)
D
Serum TNF-α (pg/mL)
r = -0.8820;
P = 0.0478
Complement Activation (CH50)

Figure 23. Proposal for the relationship between complement activity and NK cell activation in patients after rituximab treatment

Consumption of complment activity induced by rituximab allows NK cells to become activated. Activated NK cells then induce lysis of tumor cells.


rituximab binds to B cells
C1q initiates complement cascade
Complement activity NOT consumed
Complement activity consumed
NK cell activation
Regression of tumor

CHAPTER V

GENERAL DISCUSSION

New role for complement in antibody therapy

The studies detailed in this dissertation have contributed to our knowledge of the relationship between complement and cellular-mediated cytotoxicity. Understanding the mechanisms in which mAbs induce tumor lysis will help improve the effectiveness of antibody therapy. Although there is no definitive evidence for the role of complement activity, the current view is that CDC contributes to the therapeutic effects of rituximab by acting in addition to ADCC. Numerous studies utilizing in vitro and in vivo models have demonstrated that CDC is an effective mechanism of tumor lysis (reviewed in Chapter I). However, clinical studies have yet to correlate complement activity with the outcome of patients treated with rituximab. The findings in this thesis suggest that complement activation may actually be impeding the anti-tumor effect of rituximab through inhibiting ADCC. In chapter II, I demonstrated that the presence of complement at concentrations of rituximab above 0.04μg/ml inhibits NK cell activation induced by rituximab-coated target cells. This inhibition was found to correlate with the amount of C3b deposited on the rituximab-coated targets. I've also shown that complement activation blocks the binding of NK cells to rituximab and inhibits NK cell-mediated cytotoxicity. In chapter III, I provided evidence that the inhibitory effect of complement is present in the extravascular compartment, where rituximab likely interacts with solid tumors and lymph nodes. In addition, I demonstrated that depletion of C3 enhances NK cell activation in the presence of complement and enhances the efficacy of a mAb therapy in a murine lymphoma model. In chapter IV, I demonstrated that the upregulation of NK cell activation markers and serum levels of NK cell secreted cytokines correlate with the decreased complement activity in patients following rituximab treatment. These results suggest that complement activation may be limiting the efficacy of rituximab and provide a novel role for complement in antibody therapies.

The results presented in this thesis could have significant implications in the use of antibodies for cancer therapy. If confirmed, these findings suggest that strategies to limit the inhibitory effects of complement activation may enhance the activation and function of patient effector cells. Careful titrations of mAbs, depletion of complement prior to therapy, and the use of antibodies lacking the ability to activate complement may all be appropriate methods to enhance the efficacy of antibody therapies. These strategies are practical and may be implemented in clinical trials in the near future.

Impact on patient care

Careful dosing of rituximab

Utilizing in vitro assays, I found that complement inhibits NK cell activation at concentrations of rituximab above 0.04μg/ml. This inhibitory effect was observed when serum, transudative pleural fluid, or ascites were used as the source of complement. At rituximab concentrations between 0.002μg/ml and 0.04μg/ml, activation of NK cells occured without any inhibition. This finding is consistent with the observation that higher mAb concentrations are required for complement activation than for NK cell activation. As rituximab binds to its target, NK cells are able to interact with the hinge region of the mAb though CD16 at relatively low concentrations. In contrast, binding of C1q requires the mAb to be in a high-density region. The redistribution of CD20 into high-density lipid rafts has been shown to be crucial for the ability of rituximab to induce complement activity (reviewed in Chapter I).

The observation that NK cells are activated without inhibition at lower concentrations of mAb may be clinically useful. My results demonstrate a "therapeutic window" between 0.002μg/ml and 0.04μg/ml in which NK cells are activated without complement fixation. Currently, a standard rituximab dose is 375mg/M^2, which is given weekly for one month. In chapter IV, it was demonstrated that patient serum rituximab concentration exceeds that therapeutic window after a standard dose of rituximab (Table

2). At these concentrations of mAb, it is likely that complement is being activated, thereby inhibiting patient NK cell function. One possible strategy to circumvent this inhibition is through carefully titrating rituximab. Theoretically, if patient concentrations of rituximab are achieved within the "therapeutic window", than the efficacy of the mAb will be enhanced.

A clinical trial has recently opened involving low dose rituximab. In this trial, 42 relapsed chronic lymphocytic leukemia patients will be enrolled from the University of Iowa, Mayo Clinic, and University of Virginia. These patients will be administered $20mg/M^2$ 3 times a week with additional treatments of alemtuzumab and pentostatin administered after the initial dose. The goals of this trial are to assess the safety of this regimen and determine the physiological responses in patients treated with low dose mAbs. After each time point, samples from the patients will be collected to determine serum rituximab concentration and levels of the NK cell secreted cytokines, IFN-γ and TNF-α. NK cell count and phenotypic changes in the activation markers, CD16, CD54, CD69, and CD107a will also be assessed. Lastly, complement activity in the serum will also be determined for each sample. Future trials following the outcome of patients receiving low dose rituximab will be important to determine the efficacy of this regimen.

Depletion of complement

The use of complement depleting agents was found to enhance NK cell activation in vitro and enhance a mAb therapy in vivo. Depletion of C3 using CVF abrogated the inhibitory effect of complement and allowed NK cells to be activated at concentrations of rituximab above 0.04ug/ml. CVF improved NK cell activation when serum, transudative pleural fluid, or ascites fluid were used as the source of complement. Using a murine lymphoma model, I also demonstrated that depletion of complement improves the efficacy of a mAb dependent on NK cells. Treatment with the mAb alone improved the mean survival from 28 to 42 days after tumor inoculation. Depletion of complement with

CVF prior to mAb treatment enhanced the therapeutic effect of the mAb with 50% of the mice remaining tumor free. HC3-1496, a human C3 derivitive with CVF-like activity, also enhanced the therapeutic effect of the mAb with 80% of the mice remaining tumor free. These results suggest that the use of agents to deplete complement may be an appropriate strategy to enhance mAb therapy.

In chapter IV, I provided evidence for complement activity in patients after initial rituximab treatment. These results are consistent with numerous studies demonstrating the ability for rituximab to activate the complement cascade. Even though I've demonstrated that complement fixation inhibits ADCC induced by rituximab, it remains one of the most effective mAbs used in cancer treatment. The findings in chapter IV suggest that complement activity may be consumed after mAb treatment. It is possible that the inhibition of NK cell function occurs initially, while complement activity is still present. As complement is rapidly depleted, NK cell activation occurs, leading to the observed therapeutic response. Taken together with the studies demonstrating the importance of effector cell functions, these results suggest that depleting complement activity prior to rituximab infusion may enhance cellular-mediated cytotoxicity and the efficacy of treatment.

CVF forms a stable C3/C5 convertase that is resistant to inactivation, resulting in complement depletion. These potent effects have led to the usage of CVF to deplete complement in numerous models. Unfortunately, the immunogenicity of CVF makes it unsuitable for clinical use. Several groups have now engineered a human C3 derived protein with CVF like functions. One of these proteins, HC3-1496, is being developed as a treatment for autoimmune disease. In chapter III, I demonstrated that HC3-1496 enhances the efficacy of a mAb therapy in a murine lymphoma model dependent on effector cell function. This suggests that HC3-1496 may be an effective way to deplete complement and enhance the clinical response of rituximab. Negotiations are currently

underway to develop a clinical trial for a combination therapy consisting of HC3-1496 and rituximab.

Additionally, there are several complement inhibitor proteins being developed as therapies for various inflammatory diseases. Both circulating complement regulators (C1 inhibitor, C4 binding protein, Factor H, Factor I, S protein, and clusterin) and soluble complement receptors have the potential to inhibit the complement cascade through their respective mechanisms. TP-10, a soluble complement receptor-1, has been shown to decrease C3 and C5 convertase activity and is currently in clinical trials for the treatment of men undergoing bypass surgery (202). If approved, the use of TP-10 to inhibit complement activity could become a part of other treatment modals, such as an adjuvant to rituximab.

Design of future antibodies

Clinical studies have demonstrated that effector cell mediated mechanisms predict the outcome for patients treated with rituximab. In various in vitro and in vivo studies, I've demonstrated that fixation of complement inhibits NK cell activation and ADCC. The results of these investigations suggest that limiting complement activity may enhance the efficacy of antibody therapy. With our current understanding of complement and the structure of antibodies, it is possible to design mAbs that engage Fc receptors on effector cells but lack the ability to fix complement. The use of mAbs that mediate ADCC without inducing the inhibitory effects of complement may be a more effective treatment for cancer.

The ability for mAbs to activate complement depends on their ability to redistribute its target antigen into high-density regions through a lipid-raft mechanism. Cragg and Glennie have now characterized anti-CD20 mAbs based on their ability to redistribute CD20 and induce CDC (30, 31, 131, 190). Type I antibodies behave much like rituximab and are capable of mediating complement activity. Type II antibodies have

been shown to induce more apoptosis than Type I antibodies and have reduced abilities to mediate CDC. It has been recently shown that the Type II tositumomab is more effective than the Type I rituximab in depleting B cells in several murine models, despite having the same isotype, target affinity, and half life. Whether the difference in efficacy is due to complement activity is still unclear. Currently tositumomab is approved for clinical use as an iodine 131- radioimmunoconjugate. However, it has not been humanized or tested as a mAb alone.

Recently, a type II humanized mAb against CD20 (GA101) has been engineered for clinical use (203). In addition to capitalizing on the impaired complement activity of type II mAbs, the Fc portion of GA101 was glycoengineered to have increased affinity to FcγRIII through the addition of bisected, non-fucosylated oligosaccharides on asp297 (203). GA101 was demonstrated to have superior anti-tumor activity in human lymphoma xenograph models and superior B cell depleting activity in non-human primates when compared to rituximab (203). In addition, in vitro assays demonstrate that complement does not inhibit NK cell activation or ADCC mediated by GA101-coated target cells (Weiner GJ, manuscript in preparation). Altogether, these results suggest that GA101 may be a promising new therapy for B cell malignancies. Clinical trials on GA101 will directly compare the efficacy of Type II and Type I mAbs and will help determine the relative roles of complement and cellular mediated mechanisms of cytotoxicity.

Additional knowledge of antibody structure may also be used to manipulate the functions of mAbs. The interactions between C1q and antibodies have been studied extensively and the binding site has been mapped. It has been shown that a K322A mutation on a murine IgG2a version of rituximab reduces C1q binding (190). The ability for this mutant mAb to induce CDC is greatly impaired. Other alanine substitutions at D270, P329, and P331 also reduce complement activation (48). It is likely that similar mutations performed on the human domain of rituximab will also block C1q binding. Preventing the fixation of C1q on rituximab could inhibit complement activation and

abrogate its inhibitory effects on ADCC. As growing evidence suggests that cellular-mediated mechanisms are crucial for the therapeutic effect of rituximab, mutating the C1q binding site of the mAb may be an appropriate method to improve its efficacy.

Future directions

This dissertation explores the interactions between different mechanisms of antibody therapy. The results detailed in this thesis demonstrate the direct effect of complement activation on NK cell-mediated ADCC. As described above, these findings could impact the use of antibody therapies for the treatment of cancers. However, additional studies are necessary to confirm the hypothesis presented in this dissertation. Further investigations are also needed in order for these findings to be clinically relevant and to improve the efficacy of antibody therapy in general.

Chapter II of this dissertation demonstrated that complement fixation inhibits NK cell activation induced by rituximab. It was also shown that the binding of NK cells to rituximab through CD16 is blocked by the presence of complement. However, additional mechanisms may be involved in the observed inhibition of NK cell activation. CR3 molecules are known to be present on NK cells and have been shown to bind to C3b. In addition, there is evidence that CD16 signaling may be dependent on interactions involving CR3 (136-138, 164). Whether C3b binding to CR3 interferes with this interaction has yet to be studied. Experiments assessing the activation of NK cell CD16 and CR3 signaling pathways could determine whether CR3 plays a role in the inhibitory effect described in this dissertation.

As described above, the ability to induce complement fixation varies with different mAbs. The majority of experiments detailed in this thesis involved rituximab, which has been shown to be very efficient in activating complement. Whether complement activity inhibits NK cell activation in the presence of other mAbs may be dependent on its ability to fix complement. Since trastuzumab has been shown to be very

poor at mediating CDC (34, 35), it is expected that the presence of complement has no effect on ADCC. However, there is evidence that complement fixation occurs with alemtuzumab (33). Studies utilizing the co-culture assay with alemtuzumab and other mAbs that induce tumor lysis could correlate the ability of a mAb to fix complement with the observed inhibitory effect described in this dissertation.

Chapter III of this thesis demonstrated that depletion of complement improves the efficacy of a mAb therapy in a syngeneic murine lymphoma model. It has been previously shown that the therapeutic effect of MS11G6 on mice injected with 38C13 cells is dependent on the presence of NK cells, suggesting ADCC as the primary mechanism of action (179). However, the specific involvement of Fc receptors in this model has not been examined. Experiments using mAbs to block mouse CD16/32 would confirm the role of ADCC this model. Furthermore, MS11G6, an anti-idiotype mAb with a different target antigen than rituximab, was used because of its dependence on NK cells. In vivo experiments exploring the effects of complement depletion on rituximab have yet to be done. Studies utilizing 38C13 cells expressing human CD20 could demonstrate that the efficacy of rituximab may be enhanced by the depletion of complement.

Chapter IV of this dissertation described a correlation between decreased complement activity and NK cell activation in patients after rituximab treatment. The samples analyzed in this chapter were from a study that is currently ongoing. Although most correlations are statistically significant, samples from only five patients were analyzed. Collection of samples from additional patients is necessary for the completion of this investigation. As described in Chapter IV, previous exposure to rituximab may affect the level of NK cell activation in patients following the infusion of therapy. Continued collection of patient samples following the second and third doses would further the understanding on the pharmacokinetics of rituximab. Correlating the levels of complement activity and NK cell activation with rituximab levels following each dose

may also help determine if the "therapeutic window" described in Chapter II exists physiologically. Evidence reviewed in Chapter I suggests that NK cell-mediated ADCC seems to play a significant role in the therapeutic effect of rituximab. However, patient outcome is the most important result in a clinical study. Following these patients long-term could directly correlate levels of NK cell activation with clinical response to rituximab.

Conclusion

MAb-based therapies have become an important part of treatment for various malignancies. However, there is still much to learn about the mechanisms responsible for the therapeutic responses. The investigations detailed in this dissertation have furthered our understanding of the interactions between complement and cellular-mediated cytotoxicity. The findings in this dissertation suggest that complement may play an inhibitory role in the anti-tumor activity of mAbs. Furthermore, these studies suggest that strategies to limit complement activation may lead to a more effective therapy. Continued investigations to fully understand the mechanisms of action of therapeutic mAbs will be crucial for the development of future antibodies and to step forward in the quest for the "magic bullet".

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