THE INTERACTION OF COMPLEMENT AND ANTIBODY IN THE *IN VITRO* NEUTRALIZATION OF INFECTIVITY OF *CHLAMYDIA TRACHOMATIS* SEROVAR L2

by

RAYMOND THOMAS HALL

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Department of EXPERIMENTAL MEDICINE

The University of British Columbia
Vancouver, Canada

Date Oct. 7, 1994

ABSTRACT

Chlamydia trachomatis is an obligate intracellular human pathogen responsible for important diseases, including trachoma, the leading cause of preventable blindness, infecting more than 500 million people worldwide. It is also the etiologic agent of a sexually transmitted disease causing serious sequelae such as salpingitis, leading to ectopic pregnancy or infertility.

Chlamydial infectivity of tissue culture cells can be neutralized by anti-chlamydial antibody, however, the presence of complement with antibody results in maximal neutralization. This thesis investigated the interaction of complement and anti-chlamydial antibody, and sought to provide a mechanism for the enhancement effect in complement-dependent neutralization of chlamydial infectivity. Three approaches were used to study this problem: 1) flow cytometry was optimized for detection of purified chlamydial elementary bodies, and *in situ* binding experiments were performed; 2) outer membrane protein-complement C3b complexes were extracted and immunoblotted in order to study *in situ* binding qualitatively; and 3) *in vitro* neutralization assays were used to determine the step in the complement cascade at which antibody interacts with complement in mediating neutralization of infectivity.

The flow cytometry experiments tested the hypothesis that antibody affects the quantity of C3b bound to the surface of whole elementary bodies *in situ*. The results indicated that antibody did not augment either the rate or magnitude of C3b binding,

and that complement was fixed predominantly via the alternative pathway.

Immunoblotting of outer membrane protein-C3b complexes tested the hypothesis that antibody was determining the specific C3 target proteins on the chlamydial cell surface. However, the results showed that the major outer membrane protein was the primary C3b target protein in either the presence or absence of antibody. Immunoblotting experiments were repeated with outer membrane protein-C3b complexes treated with hydroxylamine. C3b appeared to be bound by hydroxyl esters, not amino esters, to the outer membrane proteins, and this was unchanged by antibody.

The final set of experiments utilized *in vitro* neutralization assays. The results demonstrated that antibody must be present before the formation of C5 convertase, and that if antibody was added at later stages of activation of the complement cascade, neutralization did not occur. The data also showed that neutralization occurred via activation of the alternative complement pathway, and infectivity was not neutralized when the classical pathway alone was isolated. Interestingly, neutralization occurred, although to a lesser degree, when the terminal complement components C7 and C8 were missing, suggesting that terminal components are not essential for neutralization. Flow cytometry binding experiments were repeated to measure the effect of anti-chlamydial antibody on *in situ* binding of terminal components C9 and C5b-9 neoantigen. The results indicated that the presence of

antibody significantly increased C9 and, to a lesser extent, C5b-9 neoantigen binding.

In conclusion, the experiments in this thesis demonstrated that antibody mediated complement-dependent neutralization of *C. trachomatis* serovar L2 at the stage of alternative pathway C5 convertase formation. Anti-chlamydial antibody probably configures the C3b molecules in specific locations on the major outer membrane protein; however, the binding experiments were unable to detect any augmentation of C3b binding by antibody. Neutralization may occur as a result of the covalently bound complement complex on the major outer membrane protein inhibiting reorganization of the EB into an RB. The role of terminal complement components in neutralization is unclear. These data showed an increase in terminal components bound in the presence of antibody, yet neutralization occurred when C7 and C8 were excluded. It is likely that there are several mechanisms of complement-dependent neutralization, some requiring the terminal components, and others not.

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Ab	antibody
APH50	alternative pathway hemolytic 50 assay
B-Dpl	complement factor B-depleted serum
C3bi	inactivated C3b
C5D	complement C5-depleted serum
CF	complement-fixation test
CH50	classical pathway hemolytic 50 assay
CMA	chlamydia growth media with antibiotics
CR3	complement receptor 3
DEAE	diethylaminoethyl
DLK	aspartate-leucine-lysine
E-Mg	EDTA-treated, supplemented with Mg
EB	elementary body
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycine tetraacetic acid
Fab	monovalent antibody fragment by papain digestion
FITC	fluorescein isothiocyanate
GPS	Guinea pig serum

HaK	hamster kidney
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HRP	horseradish peroxidase
IFU	inclusion-forming unit
IgG	immunoglobulin G
IHS	immune human serum
KDO	ketodeoxyoctanoate
LGV	lymphogranuloma venereum
LOS	lipooligosaccharide
LPS	lipopolysaccharide
Mab	monoclonal antibody
MAC	membrane attack complex
MIF	microimmunofluorescence assay
MOI	multiplicity of infection
MOMP	major outer membrane protein
NHS	normal human serum
OMP	outer membrane protein
PBS	phosphate-buffered saline
PBS-T	PBS-Tween 20
RB	reticulate body

RGD	arginine-glycine-aspartate
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS - polyacrylamide gel electrophoresis
SPG	sucrose phosphate glucose
VD	variable domain

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This thesis is dedicated to my wife, Bonnie, and my children, Bradley and Robert.

Raymond T. Hall
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PART 1.0 INTRODUCTION AND OBJECTIVES

The microorganism *Chlamydia trachomatis* is a small, obligate intracellular bacterium unusual among procaryotes for its high carriage rate, and unique among bacteria for its dimorphic life cycle consisting of an intracellular reticulate body, and a smaller, infectious extracellular form, the elementary body (EB). In man, it is the agent of trachoma, the leading cause of preventable blindness, infecting nearly 500 million individuals worldwide (65, 80). It is also the etiologic agent of the most widespread sexually transmitted disease in North America, responsible for important urogenital sequelae, in particular salpingitis leading to ectopic pregnancy or infertility (64, 75).

With the goal of developing a vaccine against *C. trachomatis*, numerous investigators have studied the mechanism of neutralization of infectivity in both animal models and cell culture. The production of monoclonal antibodies directed against the outer membrane, in particular the major outer membrane protein, has led to the characterization of epitopes specific for genus, species, and sub-species antigenic determinants (11, 55, 69). Although neutralizing epitopes have been well-defined and monoclonal antibodies well-characterized, there is still considerable uncertainty as to the mechanism of neutralization of chlamydiae, both *in vivo* and *in vitro*. One of the challenges has been inconsistencies in neutralization of different chlamydial serovars, as well as variations in the type of tissue culture systems used.

In addition, there has been uncertainty as to the contribution of complement to neutralization. Polyclonal anti-chlamydial antibody at high titer neutralizes chlamydia, but with the addition of complement neutralization is significantly enhanced (25, 39, 42). This effect is also seen with immune serum of low antibody titer. Complement, although activated by chlamydiae, is by itself not able to maximally neutralize infectivity.

In 1992 a symposium of leading chlamydial researchers held a workshop in order to set standards and optimize conditions for in vitro neutralization of infectivity by monoclonal antibody; however, the role of complement was not addressed (9). Investigators have reported in some cases a requirement for complement in antibody-mediated neutralization (11, 23, 39), while others have shown neutralization to occur adequately without a complement substrate (53), or with cobra venom factor, a C3b analog (38). The mechanism of complement-mediated neutralization remains unanswered. Clearly, there is a need for investigation into the interaction of complement and antibody in neutralization of chlamydial infectivity.

Based on previous findings, we believe it is important to study complement-dependent antibody neutralization to answer the following questions: what are the kinetics of the activation and deposition of C3b on the chlamydial cell surface and are they augmented by immune antibody; what is the role of the terminal complement components and the effect of antibody on the formation of the membrane attack

complex; at which step in the complement cascade does antibody exert an influence on neutralization; and lastly, which complement activation pathway is involved for both deposition of complement *in situ* and for neutralization of infectivity in cell culture?

The specific aims of this thesis were to: 1) use flow cytometry to quantitate the deposition of C3b on the surface of chlamydial elementary bodies *in situ*, 2) compare the rate and magnitude of C3b deposition in both the presence and absence of immune antibody, 3) determine the contribution of each of the complement activation pathways to C3b binding, 4) use immunoblotting to define the target proteins for C3b on the outer membrane of the elementary body, and observe the effect of antibody, 5) elucidate the nature of the bond between C3b and its chlamydial outer membrane target proteins, 6) measure the terminal complement components (C5b-9) bound in situ in both the presence and absence of antibody, and 7) perform *in vitro* neutralization assays to determine the step in the complement cascade at which antibody acts, and to determine the complement activation pathway for neutralization.

Through this investigation we will provide data which will elucidate the interaction between complement and antibody in enhancing neutralization of chlamydial infectivity *in vitro*, and describe a mechanism by which complement-dependent neutralization is mediated by antibody. This information will be important to those currently developing an immunization strategy for *C. trachomatis* (8).

PART 2.0 LITERATURE REVIEW

2.1 Biology of Chlamydiae

2.1.1. Discovery and classification. *Chlamydia trachomatis*, the agent of trachoma, was first observed in infected ocular material more than 75 years ago by Halberstaedter and von Prowazek who thought these microrganisms were protozoans and named them Chlamydiaceae, or "cloaked" animals. Later, they were classified as viruses, based on the observation that they were filtrable agents, and grew in living cells (80). During the 1960s numerous studies were carried out involving biochemical, molecular, and biological techniques that led to the conclusion that chlamydiae are procaryotic organisms that parasitize eucaryotic cells (6).

At present, the Chlamydiaceae are a family of bacteria comprised of one genus, *Chlamydia*, and three species, *C. trachomatis*, *C. psittaci*, and *C. pneumoniae* (6, 45, 80). Each species contains numerous types and subtypes. *C. trachomatis* is divided into three biovars: trachoma, lymphogranuloma venereum (LGV), and mouse pneumonitis (Table 1). The trachoma and LGV biovars are often grouped together, and consist of fifteen serovars designated by letters A through L. Subspecies designations are often used for the trachoma and LGV biovars. They are groups of serovars that are immunologically related. At present 3 subspecies groups are

recognized: B complex, F-G group, and C complex.

Table 1. Differentiation of the three biovars of *C. trachomatis*

Characterisitics	Biovar		
	Trachoma	LGV	Mouse
Natural Host:Humans	+	+	-
Mice	-	-	+
Preferred Site of Infection:			
Epithelium Cells	+	-	-
Lymph Nodes	-	+	-
Lungs	-	-	+
Intracerebral Lethality in Mice	-	+	-
Conjuctivitis in Primates	+	-	-
Plaques in L cells	-	+	+
Infection Enhanced by:			
Centrifugation	+	-	-
DEAE-Dextran	+	-	-
Number of Serovars	12 (A - K)	3 (L1 -L3)	N/A
% DNA Homology with Trachoma	100	~100	30-60

(from: Moulder, 1982)

Chlamydiae are unique among bacteria in that they possess a dimorphic life cycle consisting of an intracellular and an extracellular stage (Table 2). The extracellular form, termed the elementary body (EB), is small, about 0.3μm in diameter, and metabolically inert. The EB is the infectious stage of the chlamydial life cycle, and upon attachment and entry to an appropriate host cell transforms into a metabolically active reticulate body (RB). The RB is much larger, from 0.8 to 1.2 μm, and utilizing the host cell's ATP as an energy source, divides rapidly in a membrane-bound vesicle, the inclusion body. After cell division has occurred numerous times, an unknown signal causes the RBs to condense and transform back into EBs. At this stage the host cell usually lyses, releasing hundreds of daughter EBs into the extracellular milieu. This life cycle takes approximately 48 hours for the LGV biovar and 72 hours for the non-LGV biovars.

Table 2. Comparison of extracellular and intracellular forms of *C. trachomatis*

Characterisitics	Elementary Body	Reticulate Body
Diameter, μm	0.2-0.4	0.8-1.2
Density, g/cm^3	1.21	1.18
Infectivity	+	-
Intracellular Multiplication	-	+
Intravenous Lethality for Mice	+	-
Immediate Toxicity for Cell Culture	+	-
Cell Wall Susceptibility for:		
Mechanical Stress	-	+
Osmotic Stress	-	+
Lysis by Trypsin	-	+
Trilaminar Structure of Cell Wall	+	+
Muramic Acid	-	-
Synthesis Inhibited by Penicillin	+	-
Lipopolysaccharide	+	+
MOMP	+	+
DNA	Compact	Disperse
RNA/DNA Ratio	1	3-4
Ribosomes	Scanty	Abundant
ATP/ADP Transport System	-	+
Host-free Protein Synthesis	-	+

(from: Moulder, 1982)

2.1.2. Growth and purification. Although yolk sacs were previously used to grow chlamydiae, this method has now been replaced by tissue culture employing epithelial-like cell lines, most commonly HeLa or McCoy cells (5). By growing large flasks with confluent monolayers in tissue culture, and infecting them with chlamydiae, relatively large amounts of chlamydiae can be harvested. The resulting chlamydial preparation can be purified from the cell debris by ultracentrifugation. To further purify elementary bodies from reticulate bodies it is necessary to centrifuge through gradients of increasing concentrations of Renografin, a sucrose-like gradient which allows sedimentation of EBs and RBs at different bands. This purification method will yield very pure (up to 99%) EB preparations.(10)

Recently, Hamster Kidney (HaK) cells have been used in place of HeLa and McCoy cells. HaK cells do not express the Fc receptor and so are the cell line of choice when working with antibody-mediated neutralization of infectivity assays (72), as they would not bind antibody.

2.1.3. Chlamydial pathogenesis. The sexually transmitted pathogen *Chlamydia trachomatis* is a major cause of urethritis in men, and cervicitis and salpingitis in women. It is believed that chlamydial-induced obstruction of the fallopian tubes is a major cause of involuntary infertility in North America (64). In

addition to horizontal transmission by sexual contact, vertical transmission also occurs. Neonates may acquire the organism from their infected mothers as they pass through the birth canal, contracting conjuctivitis or pneumonia.

For decades, chlamydia has been known to be the agent of trachoma, the ocular infection most responsible for blindness in developing countries around the world. In endemic areas, virtually all infants and young children are infected. The cycle of constant reinfection, with an inflammatory process mediated by an immune response to the *C. trachomatis* heat shock protein HSP60 (65), leads to a chronic keratoconjunctivitis which may, after as long as twenty-five years, result in blindness. Topical antibiotics have been employed in an attempt to control the infection, but recovery of the organism from conjunctiva, nasopharynx, oropharynx, and rectal specimens from infants with trachoma in Egypt points to a systemic syndrome with chronic reinfection negating the temporary effects of the topical antibiotics (64).

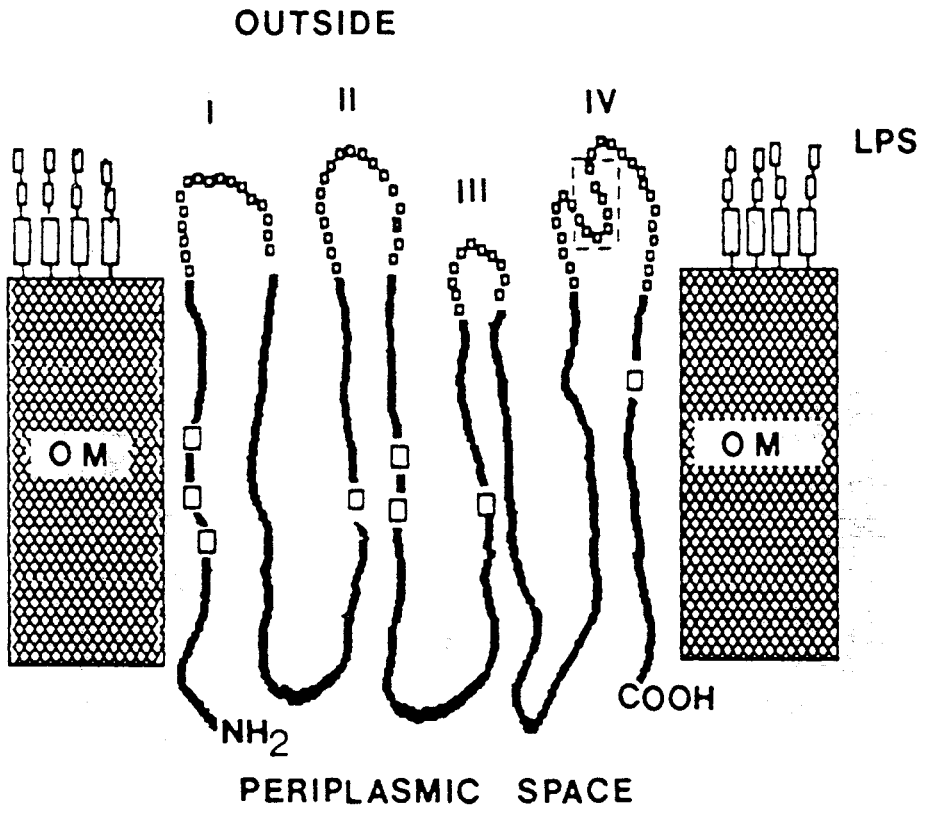
2.1.4. The Outer Membrane. The structure of the outer membrane of chlamydiae has similarities to the outer membrane of Gram negative bacteria. However, muramic acid, a component of peptidoglycan, has not been detected in chlamydiae (65). Approximately 60% of the dry weight of the outer membrane consists of a 38 - 44 kilodalton (depending on the serovar) tetrameric protein termed the major outer membrane protein, or MOMP (Figure 1) (63). MOMP is important both structurally and immunologically. It is the antigen detected in the chlamydial

micro-immunofluorescence (MIF) test, the test used for serological diagnosis of chlamydial infection, and organism typing (79). It is a transmembrane protein with four surface exposed variable segments which contain the species- and serovar-specific antigenic epitopes.

Figure 1. Diagram of MOMP in the outer membrane layer of the chlamydial cell wall.

The solid line is the transmembrane peptide chain containing the conserved cysteines (large open squares), and the small open squares represent the variable domains (VDs). The broken box within VD IV is the species-specific epitope common to all MOMPs. (LPS) lipopolysaccharide, (OM) outer membrane.

(from: Baehr 1988)



MOMP is thought to act as a porin, and also plays a role in the transformation between elementary and reticulate bodies (20). Disulphide bonds between MOMP peptides give structural integrity to the elementary body, and it has been shown that reduction of these disulphide linkages is one of the first events upon entry of the EB into a host cell.

MOMP can be recovered from sodium lauroyl sarcosine- (sarcosyl) extracted outer membrane preparations along with several other proteins, including a 60-62 kDa doublet, and a 12-15 kDa protein. When solubilized in sodium doceyl sulphate (SDS), or in reducing agents to break the MOMP disulfide bridges, only MOMP is solubilized (10). The 60-62 kDa proteins are important immunogens, which are surface-exposed on elementary bodies, and contain species-specific antigens (49). The 12-15 kDa protein is not surface-exposed, but has species and biovar specificity (86).

Two proteins, with apparent molecular masses of 18 and 32 kilodaltons, were identified on L2 elementary bodies and bound HeLa cell fragments. They were not present on reticulate bodies. The binding of these proteins to Hela cells could be inhibited by the reducing agent 2-mercaptoethanol, but not by dithiotreitol. They were not seen in the presence of protease inhibitors tosyl-phenylalanine chloromethyl ketone or tosyl-lysine chloromethyl ketone. Collectively the data suggests a role for the 18 and 32 kDa proteins in elementary body-host cell interactions (21, 81).

Both chlamydial EBs and RBs contain a lipooligosaccharide (LOS) molecule as part of their outer membranes. LOS demonstrates a positive result in the limulus lysate test, and is structurally similar to the lipid A and KDO core of *Salmonella typhimurium* rough (RE) mutants (51). It has been shown to have two antigenic sites, one shared by other bacteria, and the other genus-specific for chlamydiae (7). Acinetobacter lipopolysaccharide (LPS) has been used in the complement-fixation test for antibodies to chlamydiae, but specificty is less than 100 percent (51). Chlamydial LOS has been cloned and expressed in the outer membrane of *E. coli* (48).

2.2 Neutralization of Infectivity of Chlamydiae.

A neutralization test for *Chlamydia trachomatis* was developed in which 5% Guinea pig serum (GPS) was used to enhance neutralization by immune serum. Previous attempts at creating an *in vitro* cell culture infectivity neutralizaton assay had yielded disappointing results due to the low titers of the immune serum utilized. The addition of 5% GPS as a source of complement to this low titer immune serum increased neutralization by 100- to 1,000-fold (23). Differences in the specificities of the various immune sera to different non-homologous trachoma strains, combined with

the fact that fresh GPS but not heat-inactivated GPS enhanced protection of cell cultures by all homologous immune sera, led these researchers to deduce that the complement-fixing chlamydial group antigen was apparently not the only neutralization antigen in chlamydial strains. That is, there must be two antigens contributing to the observed neutralization event, one group antigen which fixes complement, and another strain-specific antigen not shared by all strains.

Caldwell et al. raised murine antibodies against the MOMP of *C. trachomatis* serovar L2 and neutralization studies were performed using this immune serum (11). When EBs were incubated in this serum, infectivity was reduced 50% at 1:128 dilution. When Guinea pig serum was present as a source of complement, 50% protection against infectivity occurred at a much higher dilution of immune serum, 1:2,048. This demonstrated an enhancement effect of complement in anti-MOMP antibody-mediated neutralization, although neutralization could occur to a lesser degree without complement. This study also showed that this anti-MOMP antibody did not prevent either attachment or entry into the host HeLa cell, but that neutralization occurred intracellularly. Monovalent Fab fragments of the anti-MOMP immune serum could not neutralize, indicating that cross-linking of MOMP by the bivalent whole antibody was necessary for effective reduction in the numbers of inclusion bodies in host cells. It was suggested that the cross-linking of MOMP after internalization of the EB into the host cell may prevent steps involved in

reorganization of the EB into the RB, or perhaps may act in promoting phagolysosmal fusion (11).

A study of neutralization of chlamydiae by normal human serum demonstrated a requirement of both heat labile and heat stable factors for inactivation in cell culture (25). The serum was designated 'normal' by the microimmunofluorescence test, however, more sensitive methods of antibody detection such as immunoblot were not used. When the serum was treated with Mg-EGTA to chelate calcium ions, neutralization was not observed. This suggested that neutralization was due to antibody plus classical pathway-activated complement, without the participation of the alternative pathway.

In 1984 Peeling et al used a murine monoclonal antibody specific for MOMP to neutralize infectivity in cell culture in the absence of complement. This species-specific Mab was able to neutralize two distinct serovars of *C. trachomatis*, L2 and I. There were inconsistencies, however, in this Mab's ability to neutralize under conditions of high organism count, or low antibody dilution. The latter was thought to be a prozone phenomenom perhaps suggesting a lack of effective cross-linking of MOMP when too much Mab was available for binding. This study also used a radiolabelling attachment assay to confirm Caldwell and Perry's conclusion that neutralization does not occur by preventing attachment to the host cell, but occurs at a later intracellular stage. They pretreated EBs with a neutralizing Mab or PBS and

found that the EBs associated with HeLa cells equally, rapidly, and to the same extent (55). Neutralization after entry into the host may also occur by interference with the biochemical functions of the EB, as a neutralizing Mab specific for serovar L2 MOMP abrogated ATPase activity of EBs (54).

A panel of seventeen Mabs (all directed against MOMP as determined by immunoblotting) were tested for their ability to neutralize chlamydial infectivity in cell culture. Five of seven serovar-specific Mabs neutralized, two of five subspecies-specific Mabs neutralized, while none of three species-specific nor two genus-specific Mabs had neutralizing activity. It is important to note that neutralization occurred only in the presence of complement, and controls demonstrated this to be a specific complement-mediated event (39). One of the Mabs was the same as that used by Peeling et al. in 1984 and reportedly neutralized without complement. This contradiction has not been resolved although it is likely that the explanation lies in differences in concentrations of both organism and Mab used in the two studies. Another explanation suggested by Peterson et al. is that the absence of magnesium ion in Peeling's PBS (and the presence of Mg^{2+} in Lucero's HBSS) may account for the discrepancy in results. Mg^{2+} was shown to confer protection of *C. trachomatis* infectivity against neutralization by polyclonal immune serum and complement, or Mab and complement systems. The mechanism of this protection is not known; however, it may involve an interaction between Mg^{2+} and LOS. The presence of

Mg^{2+} may strengthen bonds between the LOS molecules and require complement, and antibody, to disrupt the outer membrane.

Megran et al., in 1988, studied neutralization using donor serum categorized by microimmunofluorescence (MIF) into three anti-chlamydial antibody groups: negative (<1:8); high titer (>1:128); and low titer (from 1:8 to 1:128). The negative sera did not neutralize infectivity regardless of the presence of complement. The high titer sera neutralized equally well whether the serum was heated (to inactivate complement) or not. The low titer serum neutralized more effectively when it was unheated, that is, when complement was active. One of the low titer sera reacted on immunoblot with only a 60 kDa protein and not to the 40 kDa MOMP (42). It is possible that this serum contained reactive anti-MOMP antibodies that were unable to recognize the linear epitopes presented by the SDS-PAGE-derived immunoblot.

A study of comparative neutralization of infectivity using homologous sera showed that LGV serovar L2 is more resistant to homologous serum than is trachoma serovar E (56). Although no virulence factors were described, this intrinsic resistance to neutralization by antibody and complement of LGV compared with trachoma may be an explanation for the increased virulence and ability of LGV serovars to mount systemic infections.

A study was conducted to shed light on the mechanism of complement-antibody neutralizaton of *C. trachomatis* by isolating the alternative (C2 depletion) and

classical (factor B depletion) pathways. The results indicated that the alternative complement pathway contributed more to neutralization than the classical pathway; however, this was dependent on the relative concentration of serum and chlamydial serovar (37).

A study of the role of the terminal complement components C5b-9 in neutralization was performed by incubating EBs in C5- and C8-depleted serum. Each depleted serum neutralized better than the corresponding heated serum, indicating that complement-dependent neutralization was taking place in the absence of the terminal components. Tritium-labelled EBs were used to infect cells and heated or unheated serum was added to effect neutralization. Only incubation in unheated serum released significant radioactivity, indicating that fresh serum allows bacteriolysis of EBs, while heated serum can still neutralize, but not by bacteriolysis (37). These data, presented in 1990, have yet to be published.

Neutralization of chlamydial infectivity in cell culture by Mab specific for MOMP before host cell entry was demonstrated by Su and Caldwell in 1990. However, these experiments used divalent IgG Mabs and, therefore, aggregation could not be excluded as a possible neutralization mechanism (72). A second study in the same laboratory was performed using monovalent Fab fragments of MOMP-specific Mabs, and the results showed that neutralization by this particular Mab occurred by preventing chlamydial attachment to the host cell (73). This evidence added a new

role to the function of MOMP: that of an adhesin molecule. It also demonstrated the diversity of neutralization mechanisms possible for chlamydiae. Depending on the specificity of the Mab, neutralization has been shown to occur at many steps of the EB infectious process, both before and after invasion of a host cell.

A study of the kinetics and stoichiometry of chlamydial neutralization in HeLa cells confirmed that variations in experimental parameters have a great effect on capability of Mab to neutralize infectivity. It is important to optimize antibody concentration, incubation temperature, incubation time, and MOI of chlamydial EBs before a study employing a neutralization assay should be attempted. If not standardized, or held constant within experiments, these parameters will contribute to discrepant results between laboratories and between sets of experimental data (53).

A brief study of antibody-complement neutralization used a C3b analog, cobra venom factor, as a substitute for C3 in heat-inactivated serum. The results showed that neutralization occurred with serovar K, but not with serovar L2. This demonstrated that, in certain circumstances, late terminal complement components are not required for neutralization. Neutralization in these instances may be effected by Ab-complement complexes. This study also showed that the necessity of complement plus antibody is serovar dependent (38).

As evidenced by the preceeding summary of recent publications, there is still considerable uncertainty as to the role, or necessity, of complement in the

neutralization of *C. trachomatis*.

2.3 Biology of the Complement Cascade

2.3.1 Overview. The human complement cascade is a system of more than twenty distinct proteins acting as a constitutive nonspecific humoral defense against invading pathogens and their by-products. The complement system interacts extensively with many other defense systems, including the antibody response of B lymphocytes, the cell-mediated immune response of T lymphocytes, the inflammatory response, and the phagocytic response of leukocytes. In general, the three effects of activation of the complement system are chemotactic (attraction of leukocytes by C3a and C5a), opsonic (phagocytosis by leukocytes via C3b and C3bi receptors), and lytic (bacterial membrane disruption by the terminal complement components) (47).

2.3.2. Activation Pathways. The complement cascade may be activated by either of two pathways, the classical, or the alternative. Although discovered later, the alternative pathway is thought to be phylogenetically older, and is simpler, requiring only the interaction of a bacterial surface with the third complement component, C3. Activated C3 releases a 10 kilodalton fragment, C3a, which acts independently as an anaphylatoxin. The remaining C3b molecule then

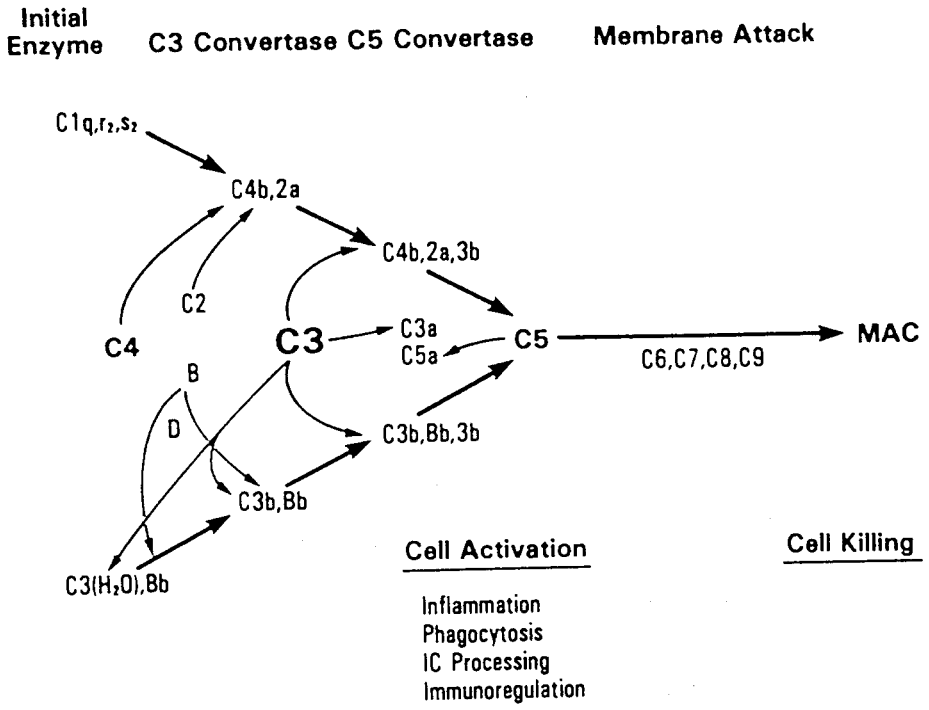
exposes an internal thioester which can bind covalently to any free hydroxyl or amino group on the bacterial surface. If C3b is able to bind, and be stabilized by cofactors, B, D, and Properdin, activation of the complement cascade begins.

Activation of complement by the classical pathway occurs when component C1q binds two molecules each of C1r and C1s. This C1 complex can then cleave C4 and C2 resulting in activation of C3. Earlier it was thought that the classical pathway was dependent on antigen-antibody complexes, in other words antibody was required for classical pathway activation, however, more recently it is being appreciated that specific antibody is not essential for C1q to bind C1r and C1s (47).

Figure 2 Schematic diagram of the complement activation pathways.

The upper left arrows represent the classical activation pathway, and the lower left arrows represent the alternative activation pathway. C3 occupies a central position in both pathways. Regulatory proteins have been omitted.

(from: Muller-Eberhard 1988)



2.3.3. Component C3. Both the classical and alternative pathways result in the formation of complexes capable of cleaving C3. These complexes are termed C3 convertases. The classical pathway C3 convertase is C4b2a, and the alternative pathway C3 convertase is C3Bb. It is important to recognize that the alternative pathway C3 convertase C3Bb contains a C3 molecule itself and is therefore a conduit for positive feedback resulting in exponential C3 increase. As C3 is constantly activating at a low level via the alternative pathway, regulatory proteins on host cells must work to avoid uncontrolled nonspecific activation. Positive and negative regulators, both soluble and cell-bound, are present at every stage of early complement component activation.

Figure 3 shows the structure of the C3 molecule. It is a heterodimer of approximately 185 kDa which can be reduced at its two disulphide linkages into two chains: the α chain, a 110 kDa molecule containing the reactive binding site, a thioester; and the β chain, a 75 kDa non-reactive fragment. The degradation of the C3 molecule has been studied extensively, and Figure 4 illustrates the various fragments commonly detected.

Figure 3. Structure of the human C3 molecule.

The numbers indicate the molecular mass in kilodaltons, except for the number at the C-terminus of the α chain and that at the N-terminus of the ß chain, which indicate the number of amino acid residues in each chain. The shaded bars together represent C3c. (I) Factor-I cleavage sites; (K) Kallikrein cleavage site in C3bi. K and the adjacent I are mutually exclusive.

(from: Muller-Eberhard 1988)

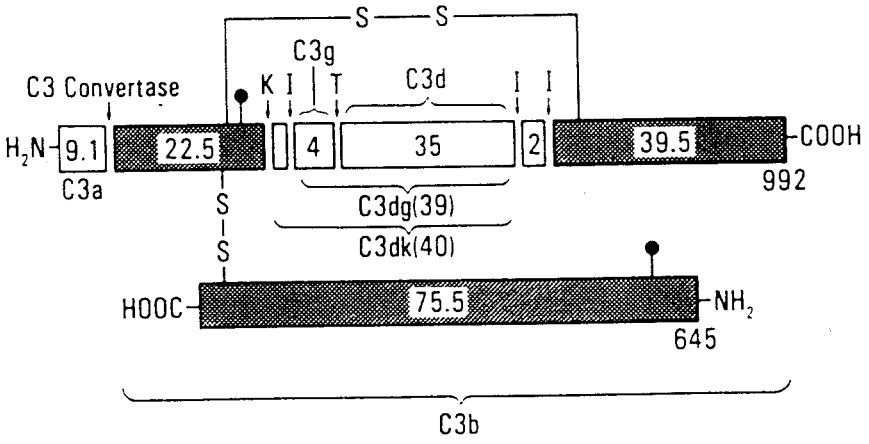
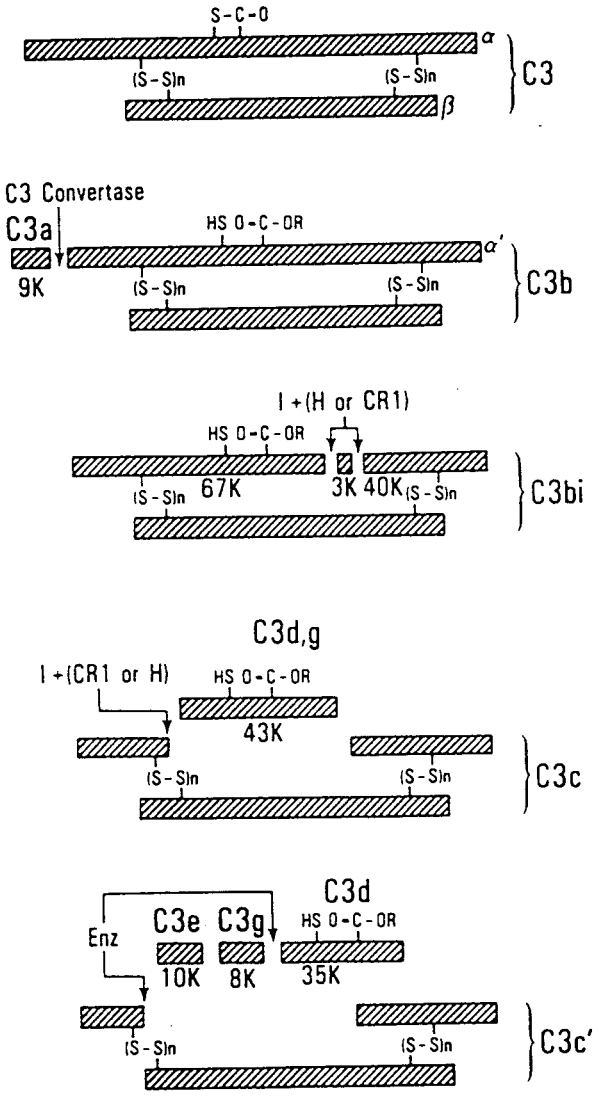


Figure 4. Degradation sequence of C3.

Upon activation of C3 to C3b, a particular fragmentation sequence occurs based on the action of negative regulators, both host- and microbe-derived. Under reducing conditions, the ß chain is separated from the α chain, and in this case C3bi can appear as a 67 kDa fragment bound to a receptive surface, as this segment contains the reactive thioester binding site.

(from: Muller-Eberhard 1988)

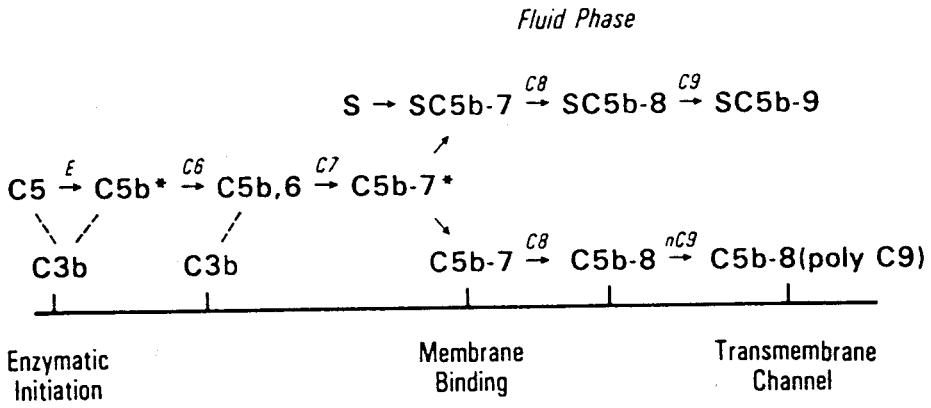


2.3.4. Late complement components C5-9. When C3b is deposited on an appropriate surface by either of the two activation pathways, the subsequent enzyme complex becomes the C5 convertase. In the case of the classical pathway it is C4b2a3b, while in the alternative pathway it is C3bBb3b. C5 convertase cleaves C5 allowing C5b to insert into the cell membrane, while the smaller fragment C5a diffuses away from the membrane acting as a chemoattractant and anaphylatoxin. Once C5b is bound to the bacterial surface, C6, C7, and C8 are added to the complex sequentially. At this point up to fifteen C9 molecules can bind to the complex, forming a tubular column which, when positioned effectively, disrupts the cell membrane enough to cause lysis of the cell. For this reason the C5b-9 polymer is termed the membrane attack complex, or MAC (92).

Figure 5. Assembly of the membrane attack complex on the surface of a target membrane.

C3b forms a covalent bond with either a hydroxyl or amino group on a target surface. C5 and C6 are complexed with C3b, but not directly with the target surface. C7 forms a stable, but noncovalent bond, and at this stage the C5b-7 complex may be bound by S-protein resulting in negative regulation. The last step, formation of poly C9 can occur with a range of C9 molecules, generally greater than 12 molecules of C9.

(from: Muller-Eberhard 1988)



2.4. Interactions of complement and microorganisms.

Several studies have shown that antibody can mediate killing of bacteria by complement without increasing the amount of complement components binding to the microorganism (28, 27). It has been suggested that antibody changes either the site, or configuration, of the terminal complement components C5b-9 on the outer surface of the target cell (26, 27, 30).

In a study of serum-sensitive and serum-resistant strains of *E. coli*, the serum-resistant strains (containing LPS with long O-antigens) activated and consumed substantially more C3, C5, and C9 than the sensitive strain which contained only short O-antigens on its LPS. It was found, however, that the terminal complement components were attached to the outer membrane on the sensitive strains in significantly larger amounts than on the resistant strains. This showed that complement resistance was not a function of a failure to activate complement, but was directly related to where the complement C5b-9 complex was able to situate itself (27). In later studies it was demonstrated that antibody assists complement in locating itself in lytically effective sites on the membrane (1, 30, 31, 71).

Activation and deposition of C3 on conidia of *Aspergillus fumigatus* was investigated using an immunoblotting technique. It was found that C3 bound to a 54- to 58-kDa surface protein by the alternative pathway, but was rapidly degraded to

smaller fragments of molecular weights consistent with iC3b, C3dg, and C3d. The surface protein on the conidia was indicated as possessing the ability to degrade bound C3 to smaller fragments as a defense against ingestion by professional phagocytes which would use C3 as an opsonin (71).

The protozoan *Tritrichomonas foetus* was studied in a viability assay based on tritiated adenine uptake. It was demonstrated that complement-preserved hypogammaglobulinemic bovine serum killed the organism moderately at high serum concentrations but with the addition of low concentrations of hyperimmune serum killing was enhanced significantly. These data indicated an interaction between complement and antibody which resulted in enhanced neutralization of the organism (1).

The elementary body of *Chlamydia trachomatis* has been shown to activate the complement cascade, and the C5a-desarginine fragments cleaved are strongly chemotactic for polymorphonuclear leukocytes (41). In a different reports, complement fragments have been demonstrated in cervicovaginal washings under non-inflammatory conditions (15). Taken together, these observations suggest that the complement component of the humoral defense against chlamydiae may have relevance in human infections (59).

Chapter 3.0 MATERIALS AND METHODS

3.1 Growth and Purification of Chlamydial Elementary Bodies.

C. trachomatis strain L2/434/Bu was used. Chlamydiae were harvested from McCoy cell monolayers by standard methods (10). Seven day old McCoy cell monolayers in 175 cm^2 plastic tissue culture flasks were emptied of growth media (CMA) and inoculated with LGV-L2 stock culture. After shaking for two hours at 37^0C the flasks were overlaid with CMA supplemented with cycloheximide and HEPES buffer. They were then incubated further for 48 hours at 37^0C and checked visually for maximal infectivity of the cell monolayer.

EBs were purified on discontinuous Renografin gradients as follows. Growth media was poured off, and 3 to 5 sterile glass beads were added to each flask. After shaking the flasks vigorously by hand to remove the cells from the plastic, the cells were pipetted several times to break up clumps, and then sonicated to lyse the cells and release the chlamydiae. Cell preparations were centrifuged at 3,000 rpm for 15 min. to pellet the cell debris and leave the chlamydiae in the supernatant. The supernatant was then layered over 35% renografin and centrifuged at high speed for 60 min. at 4^0C. The pellet contained chlamydiae, both EBs and RBs. In order to separate the EBs, the pellet from the previous spin was resuspended in phosphate-

buffered saline (PBS) pH 7.4 and layered over a discontinuous renografin gradient. It was again centrifuged at high speed (40,000 x g) for 60 min. at 4^0C and the layer containing only EBs (a mid-band) was recovered. This preparation was aliquoted into 100μl volumes and stored frozen at -70^0C.

Preparations contained >90% EBs, as determined by electron microscopy. This observation was supported by uniformity of particle size in scattering laser light during flow cytometry. Samples were found to contain approximately 4 x 10^8 IFU/ml by iodine staining of McCoy cell cultures. EBs were resuspended in PBS, aliquoted, and stored at -70^0C until needed.

3.2. Human Serum.

Blood used as a complement source was collected from healthy volunteers. The serum was separated, and aliquots were frozen at -70^0C. On the day of the binding experiments a portion of the thawed serum aliquot was tested for complement activity and found to have, on average, 200 CH50 and 146 APH50 units/ml. Western blot against chlamydial outer membrane proteins (OMP), failed to detect any antibody-reactive bands. This serum was designated normal human serum (NHS).

Immune human serum (IHS) was pooled from three donors. Frozen sera from two

patients with acute chlamydial genital infections were kindly provided by W. R. Bowie, Dept. of Medicine, Univ. of British Columbia. These sera were reactive with all 15 serovars of *C. trachomatis* as well as *C. psittaci* and *C. pneumoniae* by MIF (kindly performed by Dr. Rosanna Peeling, Chlamydia Laboratory, Laboratory Centre for Disease Control, Health and Welfare Canada). The MIF titers for serovar L2 of these sera were 1:32 and 1:8. The third serum was from a laboratory worker with no history of chlamydial infection but who has worked with LGV L2 for many years. This serum was also broadly reactive by MIF, with a titer of 1:8 for serovar L2. Two of these sera had no higher titers against the other serovars including *C. pneumoniae* TW183 and *C. psittaci* 6BC. One of the sera (reacting at 1:8 to L2) reacted at 1:32 to serovars B, D, and E, and 1:16 to serovars C, H, I, J, and A.

Western blot against serovar L2 OMP demonstrated specificity of these sera for epitopes of 8-12 proteins including MOMP, the 60 and 62 kDa cysteine-rich proteins, and other proteins at 10, 29, 32, 75, and 92 kDa. A chlamydial complement fixation test (66) using a genus-specific antigen performed on IHS yielded a titer of 1:32. A chlamydial neutralization test (9, 72) using this serum demonstrated that 10% IHS with fresh NHS as complement substrate resulted in a 99% reduction in inclusion forming units of the LGV L2 strain in HaK cells, compared to only 50% reduction with heated IHS alone.

IHS and some aliquots of NHS were heated for 30 min at 56^0C and then found to

be negative for complement activity by CH50 assay. Some aliquots of NHS were run through a Protein A-Sepharose column to remove serum IgG. This serum, called IgG-depleted NHS, was found to be negative for IgG by western blot with goat anti-human IgG antisera (Sigma Chemicals, St. Louis, MO).

3.3. Flow Cytometry

3.3.1 Measurement of C3 Binding. In order to optimize the parameters of the flow cytometer (EPICS Profile, Coulter Corporation), EBs were incubated with a fluorescein isothiocyanate (FITC)-conjugated murine monoclonal antibody to *C. trachomatis* (Kallestad, Chaska, MN) for 30 min at room temperature. After washing three times in PBS, EBs were run on the flow cytometer and the optimum parameters for detection of EBs were set and stored in the onboard computer. The flow cytometer was set to measure 10,000 particle counts, and the sheath pressure was set at maximum in order to count only those particles of approximately 300 nm diameter.

To determine the time course of binding, EBs were incubated with NHS, or NHS supplemented with 10% IHS, for 1, 5, 15, 30, 45, and 60 min at 37^{0}C with gentle shaking. Controls were incubated for 30 min. Cold PBS was added to samples to stop the reaction and samples were then held at 4^{0}C until all reactions were stopped.

Samples were washed three times with cold PBS and centrifuged at 41,000 x g for 60 min at 4^0C to pellet the EBs. To detect bound complement, samples were incubated with FITC-conjugated goat anti-human C3 (Atlantic Antibodies, Scarborough, ME), diluted 1:500 in PBS, for 30 min at 37^0C with gentle agitation. Heat-inactivated NHS was used as a negative complement control, and normal goat serum followed by FITC-conjugated rabbit anti-goat IgG was used as a control for non-specific binding of goat IgG. After being washed and centrifuged as described above, EB-associated fluorescence was quantitated by flow cytometry.

3.3.2 Determination of Complement Activation Pathway for C3 Binding. To isolate the alternative complement activation pathway, NHS was incubated in a final concentration of 20 mM EDTA for 5 min at 37^0C shaking gently. EBs were added to all samples, a final concentration of 25mM $MgCl_2$ was added to appropriate samples, and all were incubated for 30 min at 37^0C shaking gently (19). Control EB samples were incubated in either NHS or heat-inactivated NHS without preincubation in EDTA. After washing three times with PBS, bound C3 was detected by incubation in FITC-conjugated human C3 antisera as described above.

In the next set of experiments designed to isolate the classical complement pathway, EBs were incubated in complement factor B-deficient serum (Quidel, San Diego, CA) with and without heat-inactivated 10%, 20%, and 40% IHS (as a source of specific

antibody) for 30 min at 37°C, and washed three times in PBS. Complement factor B-deficient serum was tested for complement activity and found to be negative for APH50, but to have a normal CH50 value (119 units/ml). Bound C3 was detected and measured in the flow cytometer as in the previous section. NHS and heat-inactivated NHS were included as positive and negative controls, respectively.

3.5. Chlamydial Outer Membrane Proteins (OMP)

3.5.1 Extraction of OMP and OMP-C3b Complexes. Purified EBs in PBS were incubated with either NHS, NHS with 10% IHS, heat-inactivated NHS, or PBS at a 1:1 v/v ratio for 45 min at 37°C with gentle agitation. They were then washed twice in PBS, to remove excess serum and centrifuged at 41,000 x g for 60 min at 4°C to pellet the EBs. OMP was extracted by the method of Caldwell et al (10). Briefly, EBs were incubated in PBS, pH 8.0, containing 2% sarcosyl (w/v) and 1.5 mM EDTA for 60 min at 37°C, centrifuged at 100,000 x g for 60 min at 25°C, resuspended in sarcosyl and centrifuged again under the previous conditions. The insoluble pellet was washed and centrifuged three times at 100,000 x g for 30 min at 4°C in PBS, pH 8.0, and then incubated in reducing Laemmli SDS sample buffer (33) for 1 hour at 37°C before being boiled for 2 min, until solubilized. Aliquots

were frozen at -70^{0}C prior to analysis. Samples were designated OMP (outer membrane protein), OMP-C3b (OMP preincubated with NHS), OMP-C3b-Ab (OMP-C3b preincubated with NHS supplemented with 10% IHS), or OMP-heated NHS.

3.5.2 SDS-PAGE and Immunoblotting. Samples of IgG-depleted NHS and samples of OMP-complexes (described above) were electrophoresed under reducing conditions on 7.5% polyacrylamide gels according to the method of Laemmli (33). Silver-staining demonstrated protein banding patterns characteristic of *C. trachomatis* L2 outer membrane complexes (49). They were then transferred to nitrocellulose membranes by the method of Towbin (77) and immunoblotted to detect either human complement C3 (C3 probe) or the chlamydial major outer membrane protein (MOMP probe).

C3 was detected using a goat antibody to human C3 (Sigma Chemicals, St. Louis, MO, as well as Quidel, San Diego, CA) followed by horseradish peroxidase-labelled rabbit antibody to goat IgG (Sigma Chemicals). MOMP was detected using a mouse monoclonal antibody directed towards a species specific epitope on MOMP (kindly provided by I. W. Maclean, Dept. of Medical Microbiology, Univ. of Manitoba) followed by horseradish peroxidase-labelled rabbit antibody to mouse IgG (Dako Corp., Santa Barbara, CA). Control blots substituted normal goat serum (C3 probe) and normal mouse serum (MOMP probe) for the primary antibodies. An additional

control blot for non-specific binding of goat antibody included goat anti-mouse IgG antisera (Sigma Chemicals, St. Louis, MO) as a primary antibody. Blots were probed with anti-C3 followed by anti-MOMP, and also in the reverse order. On one blot a monoclonal antibody to the 57 kDa OMP2 chlamydial protein was used (kindly provided by I. W. Maclean, Univ. of Manitoba). The sensitivity of the Western blots was increased by the use of enhanced chemiluminescence (ECL, Amersham Inc.). This technique also allowed extinguishing of the first probe, followed by sequential reprobing of the same blot with a different primary antibody (ECL protocol, Amersham).

3.5.3 Hydroxylamine Cleavage of OMP-C3b Complexes. Samples containing OMP-C3b complexes were incubated for 45 minutes at 37^{0}C in freshly prepared 2M hydroxylamine, pH 10.5. Hydroxylamine was removed by dialysis overnight at 4^{0}C against 0.1% SDS in distilled water. Samples were then separated by 7.5% SDS-PAGE as previously described. Control samples were treated with distilled water instead of hydroxylamine.

3.6 Neutralization of Infectivity Assay.

3.6.1 Determination of Percent Reduction. HaK cells were grown in tissue

culture flasks using standard methods. 1 x 10^5 cells/well were inoculated into 96-well microtiter plates and grown for 24 hours at 37°C in 5% CO_2. Cell monolayers were confluent and viable. Cells were washed once in either PBS or Sucrose-Phosphate-Glutamate (SPG) and then inoculated with 50μl chlamydial EBs. EBs had been previously neutralized by incubating for 30 min at 37°C in combinations of serum (heat-inactivated IHS, NHS, or PBS) in separate reaction vessels. Serum was removed and EBs were washed twice in PBS prior to their inoculation onto the cell monolayer. The HaK cells were then incubated for two hours at 37°C on a rocking platform. The inoculum was removed, wells were washed once in PBS, and then overlaid with Chlamydia Media with glucose, antibiotics, HEPES buffer, and cycloheximide (CMGA+H+C). HaK cell plates were incubated for 42-44 hours at 37°C in 5%CO_2.

After removing the medium from the wells, they were washed once in PBS, and fixed in absolute methanol for 20 minutes at room temperature. The wells were washed once in PBS, and a rabbit anti-Chlamydial L2-MOMP antibody at a 1:500 dilution in PBS was overlaid on the monolayers. Plates were incubated for 70 minutes at 37°C on a shaker, then washed four times in PBS-T. Horseradish peroxidase conjugated goat anti-rabbit IgG (1:1000) was added to each well and the plates were incubated for 45 minutes at 37°C. After washing four times in PBS-T, HRP-4-chloronaphthol colour substrate was added and the plates were left at room

temperature until colour appeared in the positive control wells, normally after 5 minutes. HRP colour substrate was removed from all the wells and they were washed once in PBS and then resuspended in 100uL/well PBS. The plates could then be read under an inverted microscope at 200x magnification (9). Chlamydial inclusion bodies in infected HaK cells appeared solid black against a clear background of uninfected cells. Five to ten fields were counted in each of triplicate wells and the average of all fields counted was calculated. Neutralization was expressed as percent reduction IFU relative to PBS buffer control using the following equation:

$$\% \text{ reduction} = \frac{\text{IFU of PBS control - IFU of serum test}}{\text{IFU of PBS control}} \times 100$$

3.6.2 Complement Activation Pathway in Neutralization. The alternative pathway was selected by preincubating NHS for 5 min in 20mM EDTA, to abrogate both pathways, then adding back 25 mM $MgCl_2$ to restore only the alternative pathway. Neutralizaton assays performed using this serum isolated the contribution of the alternative pathway to complement activation.

The classical pathway was selected by replacing NHS with complement factor B-depleted serum (Quidel, San Diego, CA), with the addition of heat-inactivated IHS

as a source of antibody. The CH50 of the factor B-depleted serum was normal at 166 units/ml. Neutralization assays performed with this serum tested the contribution of the classical pathway to complement activation.

3.6.3 Sequence of Addition of Antibody in Neutralization. To determine the point in the complement cascade at which antibody mediates neutralization of infectivity, EBs were incubated in complement C5-depleted serum, followed by the sequential addition of purified complement components C5, C6, C7, C8, and C9. Antibody was added at three different times in the complement cascade: pre-C5D; post-C5D; and post-C5D,C5-9. EBs were washed in PBS and centrifuged at 40,000 x g twice between steps.

3.7 Measurement of C9 and C5b-9 Binding by Flow Cytometry.

EBs were incubated in NHS with and without 10% IHS for 30 minutes at 37^{0}C while shaking gently. Samples were then washed three times in PBS and incubated in primary antibody for one hour on a shaker at 37^{0}C. A goat polyclonal anti-human C9 antibody (Quidel, San Diego) was used to measure C9, and in a separate set of experiments a mouse monoclonal anti-SC5b-9 neoantigen antibody (Quidel) was used

to measure C5b-9. Both primary antibodies were used at a 1:500 dilution in PBS. After washing three times in PBS-T (0.1% Tween 20), flourescein isothiocyanate (FITC) conjugated secondary antibodies directed against goat IgG (Southern Biotechnology) and mouse IgG (Sigma) primary antibodies, respectively, were used at 1:1,000 dilution. Samples were incubated for one hour at 37^{0}C shaking. After washing three times in PBS-T, samples were held at 4^{0}C until read in the flow cytometer.

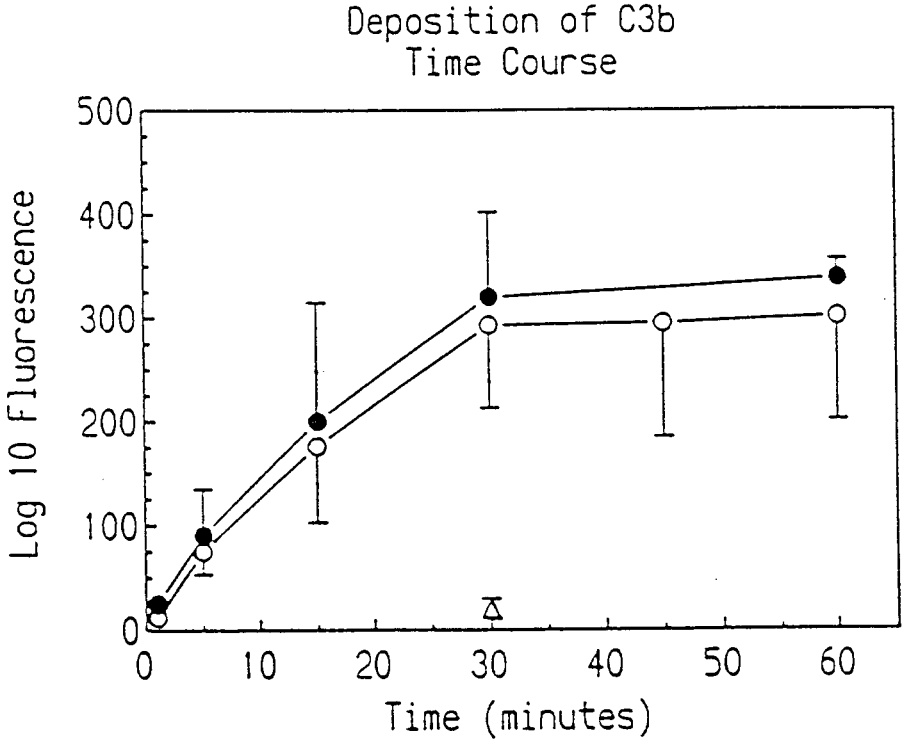
Chapter 4.0 RESULTS

4.1 Deposition of C3b on Elementary Bodies and the Effect of Antibody.

C3 binding to EBs of serovar L2 in NHS increased abruptly reaching a plateau at 30 min (Figure 6). When 10% IHS (specific antibody) was introduced neither the rate of increase, nor the level of maximal binding ($\log_{10}$ 320 $\pm$ 81.9 fluorescence units with antibody, and 292 $\pm$ 80.4 fluorescence units without antibody), were altered. Control FITC-conjugated normal goat serum showed negligible fluorescence (data not shown). EBs incubated in heat-inactivated NHS for 30 minutes had a mean fluorescence intensity of $\log_{10}$ 20.1 $\pm$ 8.2 fluorescence units indicating minimal C3b binding. Subsequent experiments using NHS preincubated in EDTA revealed no detectable fluorescence (data not shown). This is therefore a better method of completely inactivating complement in this setting than heat-inactivation.

FIGURE 6. Deposition of C3 on elementary bodies of *C. trachomatis* as determined by flow cytometry.

Elementary bodies were incubated in fresh NHS (O), NHS supplemented with 10% IHS (●), or heat-inactivated NHS (56°C for 30 minutes) (Δ). Bound C3 was detected by incubating in FITC-conjugated goat antibody to human C3. The data shown are the mean ± SD of 4 experiments.



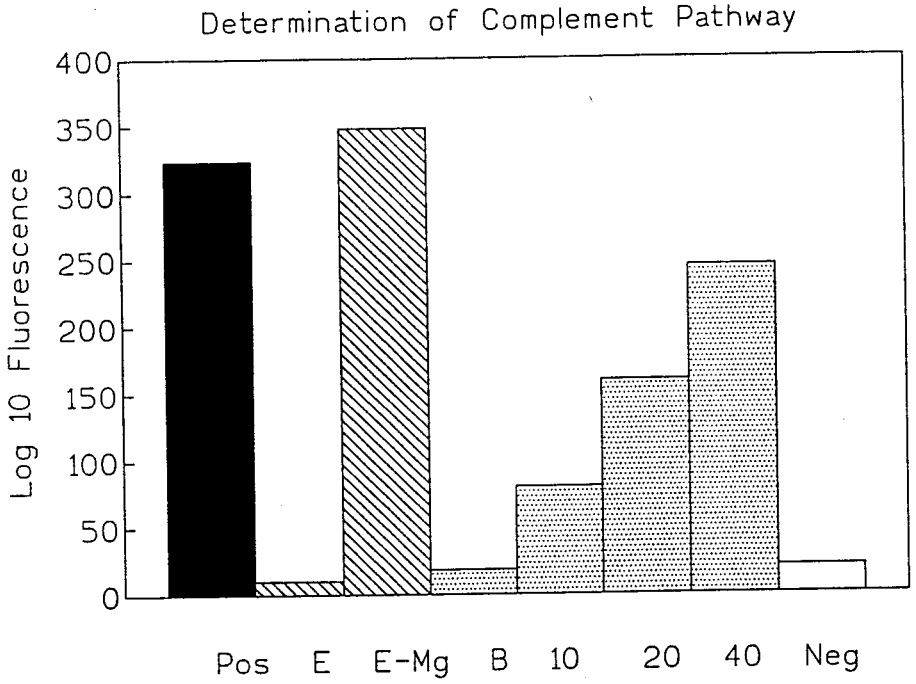
4.2 Complement Activation Pathway in C3 Binding.

Figure 7 combines the data from two sets of experiments, each designed to isolate one of the complement activation pathways and to determine its role in the deposition of C3b on chlamydial EBs. When normal serum was preincubated with EDTA, C3 bound to EBs was reduced to a minimal level. However, when the Mg^{2+} ion was added back, selectively restoring the alternative pathway, binding of C3b returned to the level of the fresh serum positive control.

To examine the contribution of the classical pathway, factor B-deficient serum was substituted for normal serum, preventing the alternative pathway from activating. Binding was reduced to the level of heat-inactivated serum. When factor B-deficient serum was enriched with 10%, 20%, or 40% heat-inactivated immune serum, to promote activation of the antibody-dependent classical pathway, binding was restored in a dose-dependent manner.

FIGURE 7. Determination of the complement activation pathway.

C3 bound to EBs was detected with FITC-conjugated goat antibody to human C3. EBs incubated in fresh NHS was used as a positive control (Pos). NHS preincubated in 20mM EDTA abrogated both complement pathways (E). Adding back 25 mM $MgCl_2$ (E-Mg) selected the alternative pathway. Substituting complement factor B-deficient serum for normal serum (B) and supplementing factor B-deficient serum with 10%, 20%, and 40% heat-inactivated IHS (10, 20, 40) selected the classical pathway. Heat-inactivated NHS served as a negative control (Neg).



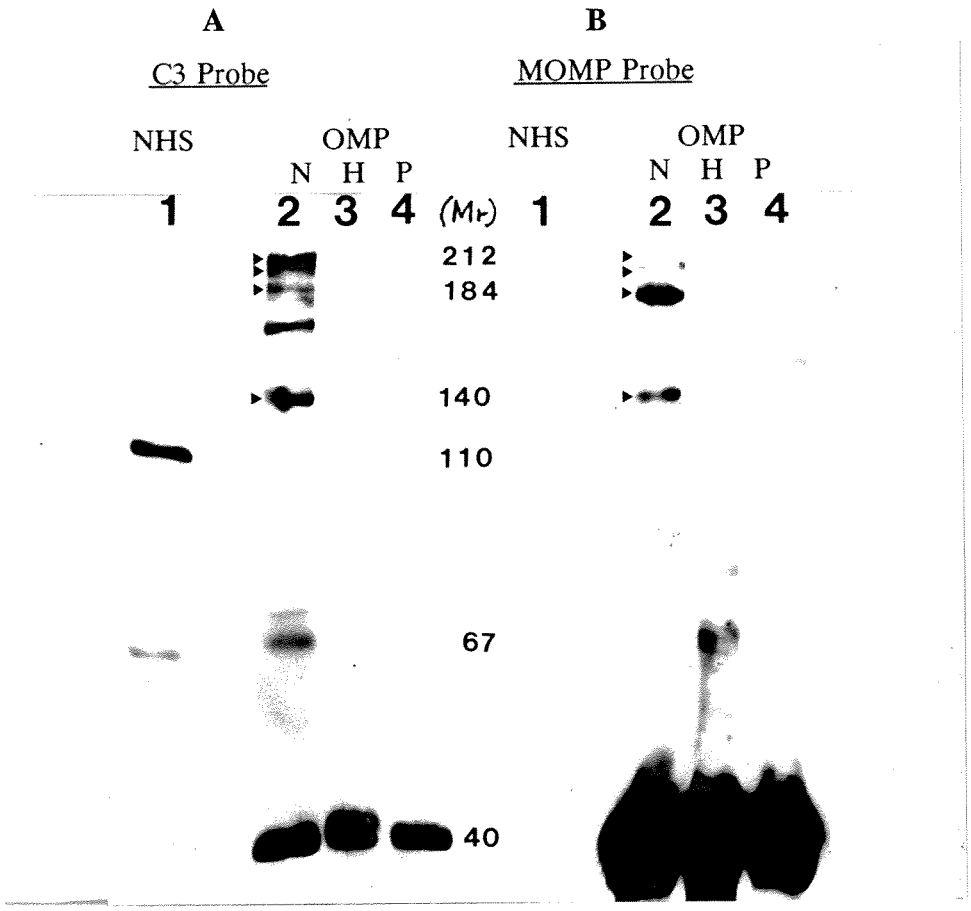
4.3 Immunoblotting of Outer Membrane Protein-C3b Complexes.

Extracted OMP-C3b complexes were electrophoresed under reducing conditions on 7.5% polyacrylamide gels and transferred to nitrocellulose membranes. Probing with goat antisera to human complement C3 α-chain revealed protein bands of various apparent molecular weights (Figure 8A). Lane 1, IgG-depleted NHS, demonstrates bands of 110 and 67 kDa: the α-chains of C3 and C3bi, respectively. On blots probed with anti-C3 reactive with both α and ß-chains, the ß-chain was also seen at 75 kDa (data not shown). In lane 2, anti-C3 antibody detected OMP-C3b complexes at several molecular weights. As the α-chain of C3b normally migrates at approximately 100 kDa (after cleavage of the 10 kDa C3a fragment), the higher MW bands, seen at 140, 166, 184, 202, and 212 kDa represent binding complexes of chlamydial proteins and C3b. Other bands detected at 40 kDa in lanes 2, 3, and 4 are discussed below. Figure 8B shows the same blot as in Figure 3A, sequentially reprobed with a MAb to MOMP after extinguishing the C3 probe. Blots exposed after being extinguished showed no bands prior to reprobing. Lane 1, IgG-depleted NHS, does not cross-react. Lanes 2, 3, and 4 (OMP-C3b, OMP-heated NHS, and OMP respectively) show intense bands at approximately 40 kDa, illustrating MOMP in its uncomplexed state. Lane 2 alone, OMP-C3b, reacts with bands of 140, 184, 202, and 212 kDa corresponding to the MWs of four of the five bands detected with

the anti-C3 antibody. The MOMP of *C. trachomatis* possesses a molecular mass of approximately 40 kDa (10), and covalently bound to the α-chain of C3b would migrate at 140 kDa. The bands seen at 184, 202, and 212 kDa may contain multimers of MOMP bound to C3b. These protein complexes, which line up at identical MWs when the original ECL films are superimposed, have bound antisera to both C3 and MOMP, in both cases at MWs higher than their native states, but consistent with complexes of C3b α-chain and MOMP or multimers of MOMP. Probing the blots first with anti-C3 antibody then with anti-MOMP antibody, or in the reverse order, did not affect the result.

FIGURE 8. Detection of chlamydial outer membrane protein-C3b complexes by immunoblot.

Elementary bodies were incubated in either serum or PBS and the outer membranes were extracted as described in Materials and Methods. Panel A was probed with an antibody to C3. Lane 1, NHS, demonstrates the alpha chain of C3 at 110 kDa and a 67 kDa fragment of C3bi. Lane 2 represents the alpha chain of C3b bound to outer membrane proteins. Lanes 3 and 4 show EBs incubated in heat-inactivated serum and PBS, respectively. Bands at 40 kDa in lanes 2, 3, and 4 were unexpected and are discussed in the text. After extinguishing the C3 probe, the same blot was reprobed with a MAb to the chlamydial MOMP (panel B). Lane 2 demonstrates four of the five complexes seen in panel A (▸). Lanes 2, 3, and 4 all demonstrate the intense signal of native MOMP at 40 kDa.



Results

The binding complex appearing at 166 kDa in the C3 probe (lane 2, Figure 8A) is unidentified, as it did not cross-react with a MAb to MOMP nor to a MAb to the chlamydial 57 kDa OMP2 (data not shown). In Figure 8, panel B, the MOMP probe, lanes 3 and 4 (OMP-heated NHS and OMP) do not react other than at 40 kDa, as expected since these samples were preincubated in heat-inactivated serum and PBS, respectively (the band at 70 kDa, lane 3, was seen inconsistently and is probably due to a multimer of MOMP, and the faint band seen at 150 kDa, lane 4, was seen only in this particular blot). However, in the C3 probe, a reactive band was also present when OMP preparations were blotted with the C3 probe, even when there was no apparent source of C3 (Figure 8, panel A, lanes 3 and 4). A normal goat serum control blot, as well as a goat anti-mouse IgG control blot, did not show bands at 40 kDa, or elsewhere (data not shown). The normal mouse serum control blot (MOMP probe control) showed only faint bands at 40 kDa (data not shown), most likely representing nonspecific binding of IgG by MOMP as has been previously shown (49).

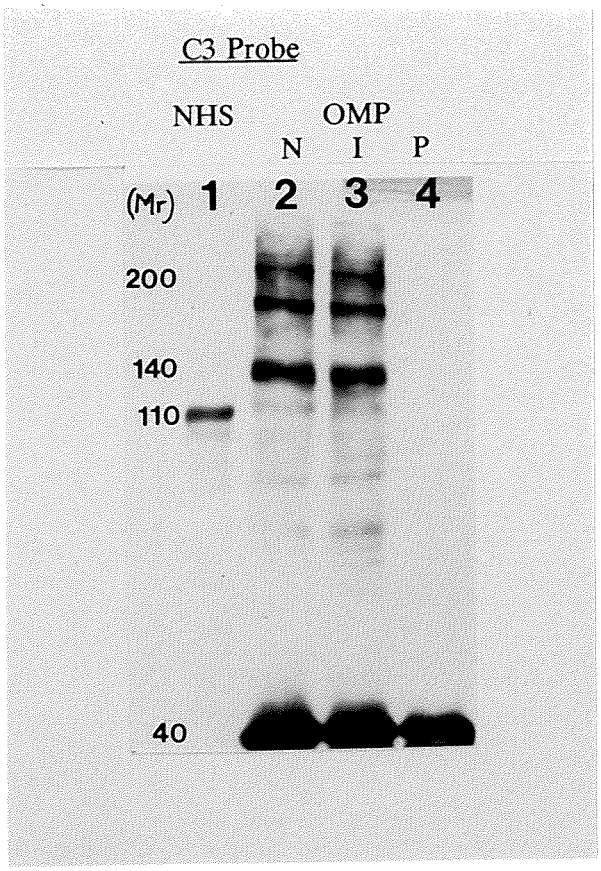
Using previously published amino acid sequences we looked for homology between serovar L2 MOMP (2, 83) and human complement C3 (18). Of interest was an aspartate-leucine-lysine (DLK) sequence that appeared in both the variable domain 3 of MOMP and the α-chain of C3. The significance of this finding has yet to be determined.

Results

Immunoblots of OMP-C3b-Ab (EBs preincubated in NHS supplemented with 10% IHS before OMP extraction) were probed with anti-C3 and anti-MOMP as in the previous experiment, with OMP-C3b lanes included for reference. Results (Figure 9) demonstrated identical binding patterns in either the presence or absence of specific Ab.

FIGURE 9. Chlamydial outer membrane protein-C3b complexes in the presence of immune serum.

Elementary bodies were incubated in either normal human serum (lane 2) or normal human serum supplemented with 10% immune serum (lane 3). The outer membrane complexes were immunoblotted with an antibody directed against human complement C3. Lane 1 is normal human serum and lane 4 is outer membrane protein in the absence of serum. The bands at 40 kDa in lanes 2, 3, and 4 are discussed in the text.

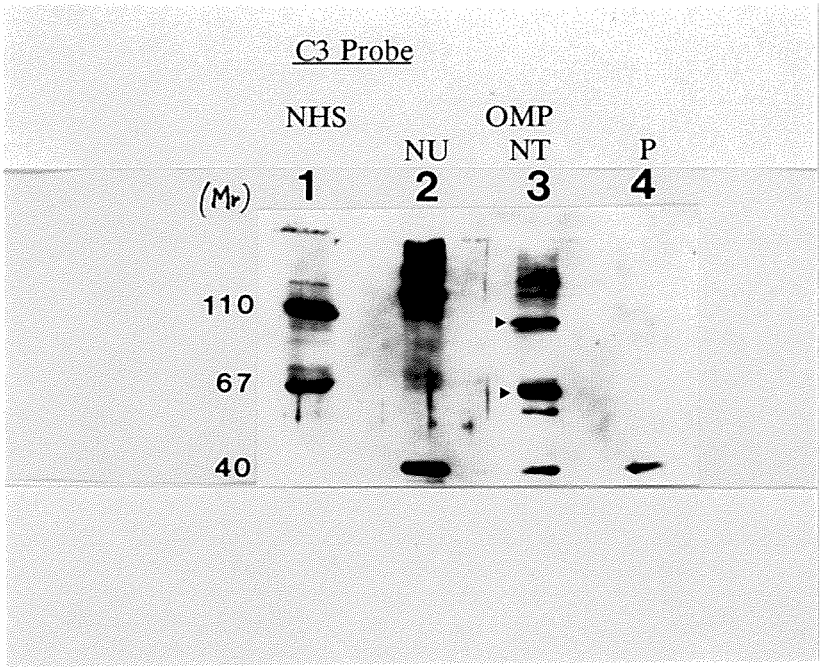


4.4 Hydroxylamine Cleavage of OMP-C3b Complexes.

To determine if the reactive thioester of C3b had bound to free hydroxyl or to amino groups, we attempted to dissociate the OMP-C3b complexes with 2M hydroxylamine. Figure 10 shows an immunoblot probed with antisera to human complement C3. Lane 1 (NHS) demonstrates reactivity with the α-chain of C3 at approximately 110 kDa, and a 67 kDa α-chain fragment of C3bi (as described above, Figure 8A). In lanes 2 and 3, the OMP-C3b complexes were treated exactly the same except that in lane 2 distilled water was substituted for hydroxylamine. Lane 2 demonstrates the high MW OMP-C3b binding complexes seen previously. Lane 3, hydroxylamine treated, shows greatly diminished high MW binding complexes along with the appearance of strong bands at approximately 100 kDa, the α-chain of C3b, and 67 kDa, a fragment of the α-chain of C3bi containing the C3b binding site. Residual high MW binding complexes are still seen faintly in lane 3 suggesting minimal binding of C3b by hydroxylamine resistant imidoester bonds, or incomplete cleavage by hydroxylamine. The majority of binding appears to be due to C3b binding to free hydroxyl groups, resulting in hydroxylamine sensitive ester linkages (36). These experiments were also repeated with OMP-C3b-Ab and results (not shown) indicated no change when binding occurred in the presence of specific antibody.

FIGURE 10. Hydroxylamine cleavage of the outer membrane protein-C3b complexes.

Complexes were incubated for 45 minutes in 2M hydroxylamine, electrophoresed on 7.5% polyacrylamide gels, and probed with an antibody to complement C3. Lane 1, NHS, shows the alpha chain of C3 at 110 kDa and a 67 kDa fragment of C3bi. Lanes 2 and 3 are samples of OMP-C3b complexes untreated (lane 2) and treated (lane 3) with hydroxylamine. Lane 2 demonstrates the high mol wt complexes described previously. Lane 3 reveals greatly diminished high mol wt complexes, along with the appearance of the alpha chain of C3b at 100 kDa and a 67 kDa fragment of the alpha chain of C3bi (▸). Lane 4, OMP alone, shows no binding complexes. The 40 kDa bands in lanes 2, 3, and 4 were unexpected and are discussed in the text.



4.5 Complement Activation Pathway in Neutralization.

Neutralization by antibody and serum with intact alternative pathway only (EDTA-Mg) was equal to antibody and fresh NHS (89.2% reduction, EDTA-Mg, cf. 81.4% reduction, NHS). However, antibody and serum with intact classical pathway only (B-Dpl) failed to neutralize infectivity (19.4 $\pm$ 2.7 IFU/200x field, B-Dpl, $p < 0.001$ cf. EDTA-Mg or NHS). The B-Dpl IFU counts were comparable to the non-neutralizing PBS control (Figure 11).

4.6 Sequence of Addition of Antibody in Neutralization.

In Figure 12, only antibody added before C5D (Pre) resulted in neutralization (79.8% reduction), whereas the addition of antibody at later steps, after C5D (Post1), and after C5D/C5-9 (Post2) did not neutralize ($p < 0.01$ and $p < 0.05$ respectively cf. pre-C5D). Each of the three stages included a control sample missing complement components C7 and C8. These controls tested the necessity of the membrane attack complex in neutralization. When antibody was present before C5D (Figure 12, Pre) neutralization also occurred when C7 and C8 were missing (54.1% reduction), although less effectively than with all terminal components C5-9.

4.7 Binding of C9 and C5b-9 and the Effect of Antibody.

EBs were incubated in fresh NHS with and without 10% heat-inactivated IHS as a source of antibody. Flow cytometry revealed C9 had bound to EBs in fresh NHS alone, however, the addition of antibody increased fluorescence significantly (151.9 $\pm$ 22.4 NHS cf. 199.4 $\pm$ 27.3 NHS+10% IHS, $p<0.01$). Similarly, when a murine monoclonal antibody directed against C5b-9 neoantigen was used, fluorescence was greater when antibody was present (31.1 $\pm$ 5.2 without antibody cf. 39.2 $\pm$ 6.4 with antibody, $p<0.05$).

Figure 11. Determination of complement activation pathway in neutralization.

Serum was treated to isolate the alternative pathway by EDTA chelation and $MgCl_2$ (E-Mg), or the classical pathway by use of factor B-depleted serum (B-Dpl). Only E-Mg neutralized infectivity, and was comparable to the untreated fresh serum control (NHS). B-Dpl did not neutralize, allowing infectivity to occur at levels equivalent to the non-neutralizing control (PBS). The data shown are the means ± SD for four experiments in triplicate.

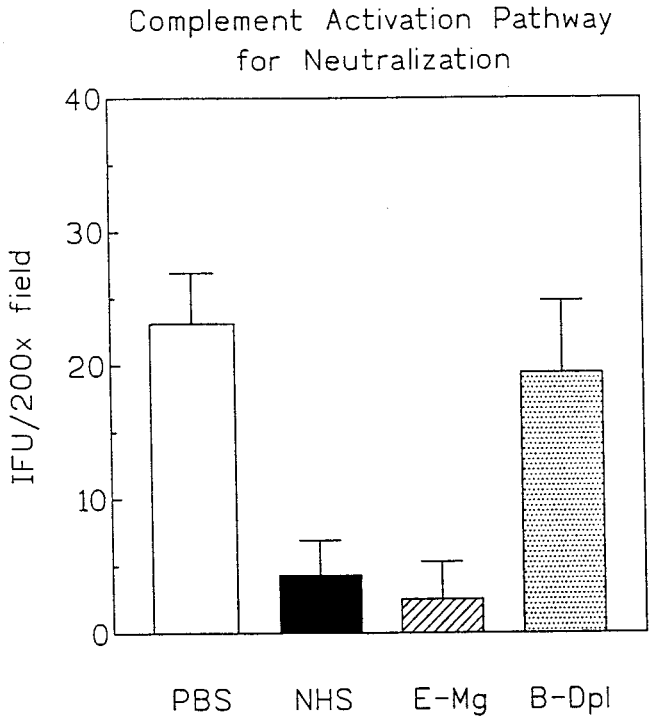


Figure 12. Sequence of addition of antibody and complement in neutralization.

Anti-chlamydial antibody was added at three steps in the complement cascade in separate reaction vessels in C5-depleted serum (C5D), followed by the addition of the missing components C5-9 (diagonal bars). Each step contained a control in which only C5, 6 and 9 were added back (speckled bars). Only antibody added before C5D (Pre) resulted in neutralization, which was comparable to the fresh serum control (NHS). Antibody added after C5D (Post1), or after C5D and C5-9 (Post2) did not neutralize. Infectivity for Post1 or Post2 were similar to the non-neutralizing control (PBS). The data shown are the means ± SD of three experiments in triplicate.

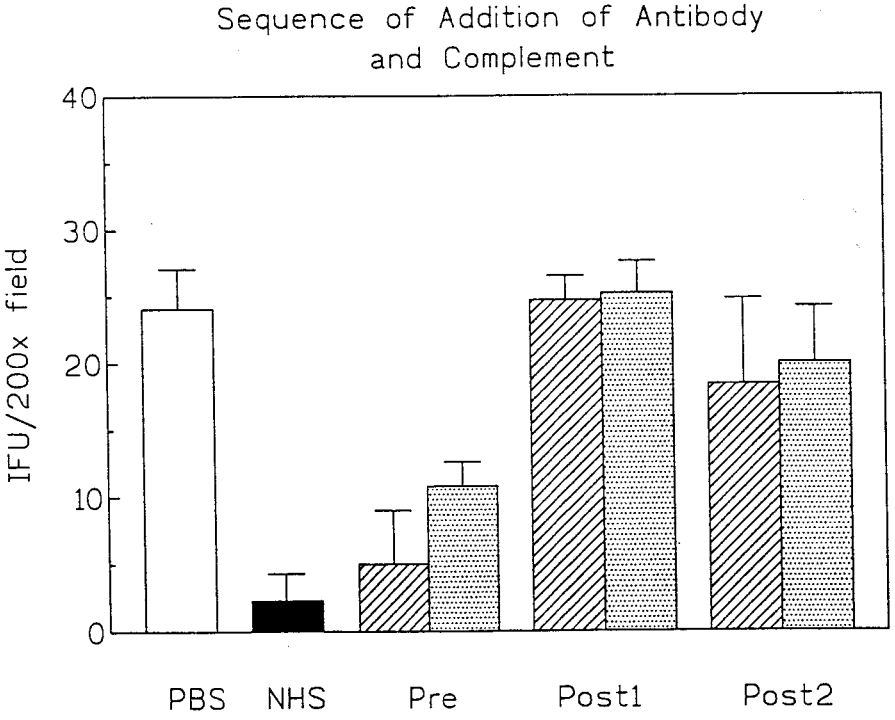


Table 3. Binding of terminal complement components to Chlamydial elementary bodies *in situ*. Flow cytometry was used to quantitate binding of C9 and C5b-9 neoantigen to whole elementary bodies in separate experiments. In all samples fresh NHS was used as a source of complement. Ten percent heat-inactivated IHS was used as a source of anti-chlamydial antibody.

Complement component	*Mean Log_{10} Fluorescence ± SD		Student's t-test
	Without Ab	With Ab	
C9	151.9 ± 22.4	199.4 ± 27.3	$p<0.01$
C5b-9	31.1 ± 5.2	39.2 ± 6.4	$p<0.05$

* four experiments in duplicate

Chapter 5.0 DISCUSSION

This study investigated the interaction of anti-chlamydial antibody and complement in the *in vitro* neutralization of infectivity of *C. trachomatis*. The flow cytometry experiments tested the hypothesis that anti-chlamydial antibody augmented binding of C3b to EBs; however, the data demonstrated that neither the rate nor magnitude of C3b binding were altered by the presence of antibody. C3 appears to be activated predominantly through the alternative complement pathway, and these results are consistent with previous studies in which alternative pathway-mediated neutralization of different *C. trachomatis* serovars is more efficient than the classical pathway, especially at higher serum concentrations (37).

Chlamydial lipooligosaccharide (LOS) may be the initial complement-activating moiety, but this is as yet unclear. Probes of intact EBs with colloidal gold-labelled LOS Mab failed to demonstrate any surface-exposed LOS epitopes (16); however, an earlier study by Maclean et al (40) reported a surface-exposed protein at 10 kDa that was presumed to be chlamydial LOS. Caldwell and Hitchcock have also described a surface-exposed MAb to chlamydial LOS (12).

Following our flow cytometry results, we thought that anti-chlamydial antibody could be having a qualitative effect on C3b binding, and so tested the effect of antibody on the array of outer membrane protein-C3b complexes. Immunoblotting

experiments revealed that the pattern of OMPs binding C3b was unchanged by the presence of antibody. MOMP appears to be the major target protein for C3 binding as determined by immunoblots with anti-MOMP Mabs; however, other outer membrane proteins may also be involved, as an additional OMP-C3b complex was detected that did not react with the MOMP or 57kDa protein MAb probes. The binding sites are likely in the surface exposed variable domains. Amino acid mapping has revealed residues with free hydroxyl groups in all four variable domains (2, 83). These free hydroxyl groups seem to be the major binding sites for C3 on EBs as evidenced by disruption of the binding by hydroxylamine.

An unexpected and intriguing finding is the binding of goat anti-human C3 antibody to a 40 kDa EB protein after incubation of EBs in PBS, although the intensity of the band was less than after incubation in fresh serum. Normal goat serum and the goat anti-mouse control antibodies did not react with this protein. If this band appeared due to nonspecific binding of goat IgG by MOMP, binding would also be expected to be seen with these goat antibody controls. This was indeed the case with the control for the MOMP probe, normal mouse serum, although the intensity was faint compared to the mouse MAb against MOMP. As this observation with the C3 probe was made consistently, the binding of anti-C3 at 40 kDa raises the possibility of C3-like antigenicity in MOMP. The possibility that this binding might be due to a degradation fragment of bound C3bi comigrating at 40 kDa with MOMP

is discounted by the fact that this observation was made in the OMP lane also, i.e., chlamydial EBs preincubated in PBS, not serum. Thus, we interpret this to suggest the possibility of antigenic mimicry of C3 in MOMP. The finding of the identical amino acid sequence DLK in the variable domain 3 region of L2 MOMP and in the α-chain of C3 and C3bi may or may not be relevant to this observation. This sequence has positively and negatively charged amino acids flanking a neutral amino acid, and in this aspect is similar to the RGD sequence which binds to the CR3 receptor (82).

The flow cytometry and immunoblotting experiments described above investigated *in situ* binding, and did not address the roles of complement and antibody in neutralization of infectivity. We subsequently performed *in vitro* neutralization experiments, and the results suggest a mechanism of interaction between antibody and complement. These data show that although the presence of neutralizing antibody increased EB binding of both C9 and polymerized C5b-9, the sequence of addition experiments indicate that complement-dependent neutralization occurs at stages prior to formation of the membrane attack complex. These findings suggest that antibody acts to mediate neutralization at the stage of C5 convertase formation and that if antibody is not present at this crucial step, complement-dependent neutralization does not occur. Antibody is likely localizing C3b (26, 27), and therefore the alternative pathway C5 convertase C3bBb3b (28, 32), at strategic points on the EB surface

allowing effective neutralization to occur. Neutralization may occur as a result of the covalently bound complement complex inhibiting reorganization of the EB into an RB. This mechanism appears to be independent of the membrane attack complex, as neutralization occurred even when C7 and C8 were missing. This observation is consistent with a recent study in which fresh C5-deficient sera, or fresh C8-deficient sera, neutralized chlamydial infectivity better than the corresponding heated sera (38), demonstrating neutralization in the absence of intact membrane attack complex.

The mediating effect of antibody resulting in neutralization may be more subtle than our flow cytometry experiments were able to detect. The data from the *in situ* flow cytometry and immunoblotting work suggest that the binding of C3b to the MOMP was unaffected by the presence of antibody. However, antibody may be configuring C3b for effective neutralization at specific locations within the variable domains of the MOMP (58, 68). From deduced amino acid sequences of MOMP (2, 83), it is apparent that all four putative surface-exposed variable domains contain hydroxyl or amino groups, which may be available to bind C3b. Although not directly supported by the data presented here, one can speculate that only particular C3b binding sites within the variable domains allow configuration of C5 convertase resulting in neutralization by preventing reorganization of the EB into an RB. The reduction of disulphide linkages between MOMP molecules is believed to be one of the initial events in the transformation of the EB to an RB. The binding of

complement complexes at particular sites on MOMP may disrupt this transformation event, resulting in neutralization.

It is likely that there are several mechanisms of complement-dependent neutralization, some requiring terminal components and others not. It also seems that the contribution of complement to neutralization may be serovar specific (4, 56). Our data here have indicated a role for antibody in complement-dependent neutralization at the formation of alternative pathway C5 convertase. Further study is needed to determine the contribution of terminal components in the neutralization of infectivity of *C. trachomatis*, and to define the exact mechanism of the complement-dependent neutralization event.

Chapter 6.0 CONCLUSION

In conclusion, the results of this study have elucidated a mechanism of interaction between complement and antibody in the *in vitro* neutralization of infectivity of *C. trachomatis* serovar L2: 1) The data of the C3b binding experiments indicate that antibody does not augment the rate nor magnitude of deposition of C3b on EBs *in situ*, nor is the target protein for C3 (primarily the MOMP) changed by the presence of antibody. 2) Despite the requirement for antibody in neutralization, the isolation of the two activation pathways revealed that C3b was bound predominantly through activation of the alternative pathway. Neutralization of infectivity in HaK cell monolayers was also mediated through the alternative pathway. Furthermore, neutralization did not occur at all when the alternative pathway was abrogated and only the classical pathway was selected. 3) The sequence of addition of antibody experiments show that antibody is required before the formation of the alternative pathway C5 convertase (C3bBb3b) in order for neutralization to occur. If antibody is introduced at any time after the formation of C5 convertase, neutralization does not occur and infectivity procedes normally, equivalent to non-neutralizing controls. The antibody-mediated neutralization at the C5 convertase step is slightly less effective, but nevertheless apparent, when terminal components C7 and C8 are excluded. This suggests that the membrane attack complex is not essential for neutralization. 4) To

test the effect of antibody on terminal components, *in situ* binding experiments were performed and revealed that the presence of antibody increased the amount of both C9 and polymerized C5b-9. This observation cautions against ruling out a role for the terminal complement components in neutralization. Further study on the role of the terminal components in neutralization is needed.

In summary, it should be noted that the experiments in this thesis were conducted with only a single strain of *C. trachomatis*: biovar LGV, serovar L2. The results of this study should therefore be read with the understanding that other serovars of *C. trachomatis* may not interact in an identical manner. It may be that differences in interaction with complement and antibody account for the more invasive nature of the LGV biovar compared to the trachoma biovar. It could be very revealing to repeat this study with a panel of trachoma serovars.

Together, the *in situ* binding experiments and *in vitro* neutralization assays presented here give a picture of the interaction of complement and antibody in the neutralization of infectivity of *C. trachomatis* serovar L2. It was beyond the scope of this thesis to test other serovars in the same manner, consequently, the larger question of differences in interaction with complement and antibody among serovars as an explanation of the more invasive nature of the LGV biovar remains to be addressed.

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