CHARACTERIZATION OF CHLAMYDIA TRACHOMATIS ANTIGENS

A Thesis Presented to the

Department of Medical Microbiology

Faculty of Medicine

University of Manitoba

In Partial Fulfillment of

the Requirements for the Degree

Doctor of Philosophy

By

© Ian William Maclean

1988

ISBN 0-315-48028-9

CHARACTERIZATION OF CHLAMYDRIA TRACHOMATIS ANTIGENS

BY

IAN WILLIAM McLEAN

A thesis submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the requirements
of the degree of

DOCTOR OF PHILOSOPHY

ACKNOWLEDGEMENTS

I would like to acknowledge the strong support given to me by my family and friends during the term of this research project. A special thanks to my advisor, Bob Brunham, for his continual support as a scientist and friend. Also, a thank you to Rosanna and Jackie for their help in the laboratory and to Carol and Paul for their help in putting this thesis together.

TABLE OF CONTENTS

Page

Page

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

A study of the Chlamydia trachomatis antigens recognized by the human immune system during a natural chlamydial infection was undertaken. Thirteen different monoclonal antibodies (MAbs) were raised to eight chlamydial antigens. Six reacted with the major outer membrane protein (MOMP) and two of these had neutralizing ability. A single MAb was produced to each of the antigens of MW 10 Kd (LPS), 29 Kd, 32 Kd, 57 Kd, 60 Kd, 70 Kd and 75 Kd. Using the MAbs and monospecific polyclonal sera, the MOMP, 10 Kd, 29 Kd and 75 Kd antigens were shown to be surface exposed. The polyclonal sera against the MOMP and 75 Kd antigen were also found to have neutralizing ability. The MOMP, LPS, 75 Kd, 70 Kd and a 17 Kd antigens released from the elementary bodies (EB) by dithiothreitol all had genus specific epitopes. The other antigens of MW 60 Kd, 57 Kd, 32 Kd and 29 Kd had C.trachomatis species specific epitopes.

The MOMP was shown to have a specific sequential breakdown pattern when whole EBs were treated with trypsin and different tryptic digestion patterns were observed when different serovars were tested. Trypsin treatment of whole EBs in combination with outer membrane isolation allowed the identification of surface exposed regions of the MOMP. Whole EBs which had been briefly treated with trypsin had enhanced infectivity when used to infect HeLa cell monolayers.

INTRODUCTION

It is perhaps a sign of the versatility and adaptability of the genus Chlamydia that its two species are the major cause of human sexually transmitted diseases in North America today and at the same time, are decimating the Koala bears of Australia with increased rates of pneumonia and infertility. The two species, C.trachomatis and C.psittaci were initially grouped together since they have the same type of morphological ultrastructure as seen during an unique life cycle. Chlamydia are intracellular parasites which grow inside a membrane bound cytoplasmic inclusion within a susceptible host cell. Inclusion containing cells were first identified in conjunctival scrapings from trachoma patients and infants with inclusion blennorrhoeae in the early 1900's.

In his address at the first Francis B. Gordon Memorial Lecture, Moulder (1974) suggested the best way to view intracellular parasitism was that of living in a hostile environment. The hostile environment referred to was the interior of the host cell. How a number of intracellular parasites have adapted to this environment was the subject of a recent review by Moulder (1985), where he outlined the major obstacles to overcome to ensure survival. These included how the parasites enter the cell and stay alive once inside, how they multiply and yet allow the maintenance of essential host cell functions and eventually how they leave and move to infect a new host cell. The process of evolution has allowed successful intracellular parasites to solve these problems using a number of different methods.

Chlamydia have two forms during their life cycle. One is the elementary body (EB), the infectious life form which in itself cannot multiply. Once inside a host cell, the EB reorganizes into the second life form known as the reticulate body (RB). The RB is metabolically active and can

multiply, but is not infectious until it changes back to the EB which is then released from the cell. Chlamydia have overcome their hostile environment and this review will try and describe how this has been done.

Chlamydia cause a wide variety of infections in both humans and animals. C.psittaci is widely distributed throughout the world, and almost any avian species can harbour the parasite. Transmission of infection from birds to man results in a febrile illness characterized by pneumonitis and systemic manifestations. C.psittaci is also associated with diseases of other animals such as abortion in goats, pigs and cattle and pneumonia in horses and cattle. However, Grayston (1986) has recently described a new C.psittaci strain known as TWAR which causes pneumonia in humans. The human diseases produced by C.trachomatis are found in a number of forms. The eye disease, trachoma, is manifested as a chronic eye infection leading to scar tissue formation and eventual blindness. It is thought to affect some 500 million people worldwide, mainly in developing countries. Lymphogranuloma venereum (LGV) is a sexually transmitted chlamydial infection rarely seen in developed countries, but is quite prevalent in parts of Africa, Asia and South America. There is initial infection of the genitals leading to a chronic infection of the nearby lymphatic tissue. The largest number of diseases due to C.trachomatis are the sexually transmitted genital infections such as non-gonococcal urethritis in men and cervicitis in women. These initial infections, if allowed to progress, can lead to more severe infections such as epididymitis, and Reiter's syndrome in men and salpingitis and perihepatitis in women. Scar tissue formation in the fallopian tubes in women can lead to infertility or ectopic pregnancy. Newborns delivered through an infected birth canal become infected and can present with neonatal conjunctivitis and pneumonia. The cost to the health care system is in the millions of dollars each year. It is therefore

advantagous to know more about this organism in order to control chlamydial disease.

The majority of information to date concerns the EB, as it is the easiest phase of the parasites life cycle to work with and it is also the infectious form of the agent. Little work has been done with the RB as it is less stable outside the cell than the EB and by the time the RB is formed, the infection is well underway. The overall driving force behind the study of chlamydial antigens is to try and eventually produce a vaccine. Grayston and Wang (1975) summarized the data showing the difficulty of vaccination in maintaining a protective effect; in some cases, vaccination has caused a more severe illness. The vaccines consisted of purified whole EBs such that the immune response may have been against a number of different antigens, some of which may not have been protective. The EB is also known to contain components which cause hemagglutination (Tamura and Manire, 1974), hypersensitivity reactions (Watkins et al, 1986) and toxicity for mice (Zhang et al, 1987). With current molecular cloning techniques, it should be possible to develop a vaccine which does not contain any of these undesirable components and yet provide full protection. This means taking the EB apart, protein by protein, trying to understand the function of each protein, in the hopes of identifying those antigens which have the potential for providing protection.

The most dominant antigens of the EB are the major outer membrane protein (MOMP) and the lipopolysaccharide (LPS). They have both been put forward as vaccine candidates against chlamydial disease. However, there are at least 15 different MOMPs so that common regions must first be found if it is to be used for a vaccine. The chlamydial LPS is genus specific but also has regions which are similar to the core LPS of gram-negative organisms. A vaccine made from this molecule might have a number of deleterious

effects. The chlamydial genome has the capacity to encode for several hundred proteins. Little is known about these other proteins and what their function is as pertains to the life cycle and the pathogenicity of this organism.

The purpose of this thesis is to identify C.trachomatis antigens (proteins) which may be directly involved in the infectious process. We produced monoclonal antibodies (MAb) against these antigens and used them in the characterization and purification of these important antigens. Both the MAbs and polyclonal monospecific serum raised against purified antigens was used to neutralize chlamydial infections in vitro. The MAbs produced against the various MOMPs were used in epitope mapping studies. This allowed us to look for common peptide fragments between the different MOMPs. Overall, these results have increased our knowledge about the EB-host cell interaction and have suggested possible proteins or peptides as vaccine candidates.

LITERATURE REVIEW

1. Taxonomy

The family Chlamydiaceae, containing the single genus Chlamydia, was formed to bring together a group of interesting organisms which caused a wide variety of diseases in a number of different animals. The common factor was an organism which grew within an intracellular inclusion during a unique life cycle. The genus Chlamydia is divided into two species, C.trachomatis and C.psittaci. In general, the two species were initially differentiated in that C.psittaci are resistant to sulfonamides and do not accumulate glycogen, while C.trachomatis are sensitive to sulfonamides and their inclusions contain glycogen. As well, C.psittaci usually infect birds or mammals other than humans while C.trachomatis is essentially restricted to human hosts, but there are exceptions in both cases.

Both species appear to have a cell wall similar to gram-negative bacteria, although a number of studies suggest they do not contain peptidoglycan. There appear to be penicillin binding proteins (PBP) (How et al, 1984; Barbour et al, 1982) which are part of the cytoplasmic membrane. This lack of muramic acid or diaminopemilic acid (DAP) in the cell envelope makes them unique among eubacteria.

Under the electron microscope, chlamydia show a hexagonal configuration of outer membrane components. Chang et al (1982) used computer processing of electron micrographs to propose a model for the outer envelope of chlamydia. They demonstrated a hexagonal lattice (unit cell) made up of six molecules surrounding a central depression. This model allowed for contact between adjacent unit cells with protein-protein interactions within the unit being stronger than those between adjacent units.

The two species of chlamydiae can be easily divided when the DNA is compared. The initial studies by Kingsbury and Weiss (1968) showed that

the base composition of C.psittaci DNA was significantly different from that of C.trachomatis. When DNA hybridization experiments were carried out, there was good homology between the two strains of C.psittaci and among the three strains of C.trachomatis, but only 10% homology between the two species. A paper by Weiss et al (1970) suggests that while C.psittaci and C.trachomatis are clearly different, the mouse pneumonitis (MoPn) strain (a C.trachomatis strain) lies somewhere in the middle having between 30% and 60% homology with human C.trachomatis strains and around 11% homology with C.psittaci.

When restriction digests of chromosomal DNA from six serovars of C.trachomatis and one strain of C.psittaci were compared on agarose gels, they showed that there were major differences between the DNA of the two species (Peterson and de la Maza, 1983). Among the C.trachomatis serovars tested, there were only slight differences in the chromosomal DNA. Palmer and Falkow (1986) have recently isolated a 7.4 Kb plasmid from C.trachomatis L2 and when used as a probe, all C.trachomatis serovars showed homologous sequences, while the slightly larger plasmids of C.psittaci and MoPn did not react. Another approach by these authors (Palmer et al, 1986) compared the Eco RI restriction patterns of 16 S ribosomal RNA genes when probed with ^{32}P-labelled 16 S ribosomal RNA. An identical Southern blot profile was seen with all tested C.trachomatis serovars while a different banding pattern was observed for mouse pneumonitis and C.psittaci strains. Of interest, when the 16 S ribosomal RNA gene was used as a probe, besides binding to C.psittaci, it also reacted with Legionella pneumophilia, Vibrio cholerae, and V.vulnificus, suggesting these genes are related and that chlamydiae are true eubacteria.

Serologic classification has been the main tool used to differentiate C.psittaci from C.trachomatis and to subgroup strains within each species.

Recent work (Perez-Martinez and Storz, 1985) showed nine different serotypes among 25 C.psittaci isolates with no significant cross-reaction with C.trachomatis. However, antigenic classification has been most successful with C.trachomatis. Using the microimmunofluorescent test (MIF), C.trachomatis can be divided into 15 serovars (Wang and Grayston, 1977). There are two main groupings with 15 distinct serovars. The two groups are the B complex (B, Ba, E, D, L1, L2, G, F) and the C complex (C, J, A, H, I, K, L3). With the advent of hybridoma technology, monoclonal antibodies (MAbs) have been produced against epitopes which have type, subspecies and species specificity. These epitopes are all found on the same macromolecule, the major outer membrane protein (MOMP). Wang et al (1985) used 15 type and subspecies MAbs in a two step typing system using MIF. There was complete correlation with the original MIF test results and in addition, three new subtypes, designated D', I' and L2', were found. A comparison of the MAb-MIF method was done by the same group (Barnes et al, 1985) with a panel of 20 MAbs (type, subspecies, species) in a dot-blot system and they found essentially 100% correlation. A MAb, which recognizes a genus specific epitope on the lipopolysaccharide of Chlamydia, reacts with both species (Caldwell and Hitchcock, 1984). Grayston et al (1986) has recently described a new C.psittaci strain which infects humans but is distinctly different than other C.psittaci isolates when a MAb is used to help differentiate the strains.

2. Attachment of the Elementary Body (EB) to the Host Cell

The chlamydial EB must first attach to the host cell before uptake and eventual replication can take place. In a laboratory where maximal infectivity is important, diethylaminoethyl (DEAE)-Dextran treated cell monolayers and centrifugation of the inoculum onto the cell monolayer are used

to enhance attachment. DEAE-Dextran (a polycation or positively charged molecule) is used to reduce repelling negative charges between the host cell surface and the surface of the EB and therefore allow greater chance of the two coming into contact. Centrifugation basically uses brute force to inbed the EB onto the surface of the host cell thereby assuring good contact, however these factors do not come into play in an in vivo situation. The unanswered question is how do EBs and host cells interact and are there specific host cell receptors and complementary ligands on the EB to allow specific receptor-mediated attachment? The literature is quite extensive on this topic (see Table 1) with many varied and often opposing views. The areas of interest have focused on carbohydrate and protein receptors on the host cell and more recently, protein ligands on the EB.

Modifications of the host cell surface have been shown to cause inhibition of attachment suggesting that N-acetyl glucosamine (NAG) and N-acetyl neuramic acid (NANA) residues are involved in attachment. For example, addition of wheat germ agglutinin (WGA), which binds to NAG, will inhibit attachment while treatment of host cells with neuraminidase to cleave off NANA residues also inhibits attachment as does addition of free NAG or NANA molecules to the host cells before infection. In general, WGA inhibits only C.psittaci and LGV serovars while not inhibiting the non-LGV strains. Neuraminidase, on the other hand, has no effect on C.psittaci 6BC and the LGV serovars, but inhibits the trachoma biovars of C.trachomatis. This suggests that there may be different receptors of attachment for the different strains.

These data might be explained by suggesting that C.psittaci may recognize a receptor which has NAG or NANA residues and so is structurally hindered when WGA is added, but these residues are not specifically important so attachment would be unaffected by neuraminidase treatment. Inhibition

TABLE 1. Comparison of effects of modification of the host cell on the attachment of Chlamydiae to the host cells.

Chlamydia		Host Cell	Wheat Germ Agglutinin	Neuraminidase	Hexoses	Trypsin	References
C.psittaci	6BC	L	Inhibition	N.D.	No Effect	N.D.	Levy (1979)
C.psittaci	6BC	L	No Effect	No Effect	No Effect	Inhibition	Hatch et al (1981)
C.psittaci	Ca1-10	HeLa	N.D.	No Effect	N.D.	N.D.	Kuo et al (1973)
C.trachomatis	L1	L	Inhibition	N.D.	N.D.	N.D.	Levy (1979)
C.trachomatis	L1	McCoy	Inhibition	N.D.	Inhibition	N.D.	Soderlund and Kihlstrom (1983)
C.trachomatis	L2	HeLa	No Effect	No Effect	No Effect	Inhibition	Bose and Paul (1982)
C.trachomatis	L2	HeLa	N.D.	No Effect	Inhibition	N.D.	Kuo et al (1973)
C.trachomatis	L3	HeLa	No Effect	No Effect	No Effect	Inhibition	Bose and Paul (1982)
C.trachomatis	A	McCoy	N.D.	N.D.	N.D.	No Effect	Lee (1981)
C.trachomatis	B	HeLa	N.D.	Inhibition	Inhibition	N.D.	Kuo and Grayston (1976)
C.trachomatis	K	HeLa	Stimulation	Inhibition	Inhibition	N.D.	Bose et al (1983)

by neuraminidase suggests that the NANA residues are specifically involved in the attachment of trachoma biovars to the host cell. To date, there is no information suggesting that there are ligands on the EB which will specifically bind free NAG or NANA residues.

The controversy of ligands of EBs continues with two recent papers. In the first report, EBs (serovars K and L1) showed less attachment to NANA deficient cell lines (Bose and Goswami, 1986). These authors also demonstrated that neuraminidase treatment would release bound EBs. However, a majority of the EBs were spontaneously dissociable, suggesting they were non-specifically bound. Allan and Pearce (1986), using serovar E on a mosquito cell line lacking NANA, showed no inhibition of binding and that neuraminidase treated McCoy and HeLa cells also had no restriction in binding. Serovar L2 also bound equally well, if not better, to a cell line lacking NAG.

Protein molecules on the host cell have also been implicated in EB attachment. Trypsinization of the host cell inhibited attachment of C.psittaci 6BC and C.trachomatis serovars L2 and L3. However, Lee (1981) observed no effect when serovar A was added to trypsinized cells.

Recent work on the components on the EB surface which mediate attachment to the host cell have identified an 18 Kd and a 32 Kd protein as potential ligands. These proteins bind to HeLa cell surface components as well as to heparin (Hackstadt, 1986a), which is known to cause the release of LGV EBs bound to the host cell. It is undecided whether these proteins are surface exposed as they were not labelled, or poorly labelled, following radio-iodination. Trypsinization of the EBs produced cleavage of the proteins which would be expected if they were surface exposed, although trypsinization has previously been shown to not reduce infectivity. Monospecific anti-sera, against the 18 and 32 Kd protein, did inhibit attach-

ment. Further work has shown these proteins to be common to all serovars, as well as C.psittaci, with minor molecular weight differences (Hackstadt, 1986b).

3. Uptake of the EB Into the Host Cell

This section will deal with the problem of how to get an EB inside a eucaryotic cell. In general, chlamydial EBs appear to be taken into a cell in much the same manner as any other particulate matter. The major difference is that the cells which EBs infect derive no benefit from the EB and cannot extrude it as it can with other engulfed material. The basic ideas of endocytosis will be reviewed and then the more specific endocytosis of chlamydia will be examined.

Eucaryotic cells have the ability to transport large molecules such as proteins and polysaccharides across the cell membrane by routes other than would be used for small polar molecules. If the cell wants to eject a protein, it is packaged in a membrane bound vesicle which fuses with the plasma membrane and allows release of the protein to the extracellular space. This process is known as exocytosis. On the other hand, the process of endocytosis is the ingestion and enclosure of a macromolecule by part of the plasma membrane to form an intracellular vesicle. The process of enclosing fluid is known as pinocytosis, while the process of ingesting particulate matter such as bacteria is known as phagocytosis.

In a multicellular organism, some cells have become specialized such that their role is to engulf large particulate matter and hence they are called professional phagocytes (Rabinovitch, 1968). These cells are the macrophages and polymorphonuclear leukocytes. Non-professional or facultative phagocytes are cells which, under normal circumstances, would not

ingest particulate matter but in certain cases, can be induced to take up material such as intracellular parasites.

The process of endocytosis is going on constantly in cells, whether they are professional or facultative phagocytes. Most cells are continually ingesting small amounts of their plasma membrane through small endocytic vesicles. A macrophage can ingest 100% of its plasma membrane within 30 minutes, with L cells (mouse fibroblasts) endocytosing at about 1/3 of this rate (Silverstein et al, 1977). The membrane being ingested must be recycled to maintain the cell surface area and volume. In some cases, the endocytic vesicles are coated with bristle-like structures on their endoplasmic surface. They are formed by the invagination of coated regions of the plasma membrane called coated pits (Goldstein et al, 1979; Pastan and Willingham, 1983). A number of proteins, as well as viruses, have been shown to enter cells via these coated pits in a process known as receptor-mediated endocytosis. The receptor for a specific ligand can be evenly distributed over the cell surface and move to the coated pit once contact is made or the receptor may be concentrated in the coated pit itself.

Once the endocytic vesicle is formed, it usually fuses with a primary lysosome to become a secondary lysosome. The lysosomes contain a number of hydrolytic enzymes which will break down the particulate matter into its base components which are then utilized by the cell. Plasma membrane components are then recycled back to the cell surface. Fusion with lysosomes is not always the end result, as some proteins are transported to internal cellular structures (Goldstein et al, 1979). How the cell determine the distribution of these structures and materials is not known.

Silverstein et al (1977) has summarized the basic properties of the endocytosis event. Particles can bind to the host cell at temperatures down to 4°C, but ingestion will not take place until a critical temperature

threshold is reached, usually around 18-21°C. Endocytosis is an energy-consuming process fueled by ATP derived from metabolism of endogenous or exogenous carbohydrates. Contractile elements such as microfilaments, made up of the proteins actin and myosin, play a major role in endocytosis. Cytochalasin B, which impairs microfilament function, also inhibits endocytosis.

Intracellular organisms must get inside a cell in order to multiply and survive. They must avoid the professional phagocytes yet trigger the endocytic reaction of facultative phagocytes. Chlamydia appears to have this ability to specify uptake into compatible host cells. Heat inactivated EBs (three minutes at 60°C) were ingested at lower levels than untreated EBs. Ingestion required specific contact between functional ligands, as unlabelled infectious EBs would not induce the uptake of labelled heat-inactivated EBs. The suggestion is that there are both specific host cell receptors and chlamydial ligands involved in the uptake of the EB into the cell. The term "parasite-specific phagocytosis" was used by Byrne and Moulder (1978) to describe the phagocytosis of chlamydia by nonprofessional phagocytes (L and HeLa cells) as compared to phagocytosis by professional phagocytes (eg., macrophages). Chlamydia were specifically taken up in greater numbers than latex beads or E.coli by L cells. Antisera were shown to prevent ingestion of chlamydia and again heat inactivation was seen, although protease treatment of the EBs did not inhibit ingestion. When ingested, chlamydial EBs are taken up in a membrane bound vesicle (Lawn et al, 1973; Friis, 1972). Adsorbtion is temperature independent but uptake is temperature dependant, optimally around 37°C (Friis, 1972). Ingestion was shown to be host energy dependent. The endocytotic vesicles containing EBs do not fuse with lysosomes, allowing chlamydial survival in a cell. Apparently, chlamydia have the ability to

prevent the normal cellular process of lysosomal fusion and degradation of the vesicle contents from taking place. Friis (1972) suggested that a specific ligand on the EB can prevent lysosomal fusion as heat-inactivated EBs, while ingested normally, did not prevent lysosomal fusion. This specific ligand might cause membrane changes in the phagosome which would be recognized or fail to be recognized by the lysosome preventing fusion, as reported by Zeichner (1983). The intact EB is not necessary to prevent lysosomal fusion, as Esseinberg et al (1983) showed that EB envelopes were able to inhibit lysosomal fusion unless they were heated and then fusion would occur. When increasing numbers of chlamydia are added to host cells, there is increased attachment and ingestion until a saturation point is met and host cell damage occurs (Byrne, 1978). This effect could be caused by no plasma membrane recycling due to lack of lysosomal fusion which would normally release membrane components.

The exact process by which EBs enter the cell is still not understood. Ward and Murray (1984) have suggested three methods which intracellular parasites such as chlamydia might utilize to enter the host cell. The first is particle stimulated endocytosis as seen in phagocytosis and the zipper hypothesis. This hypothesis suggests that as the cell engulfs the particle, it must continually be stimulated by particle ligands distributed over the particle surface or endocytosis will stop. Another method of entry suggests a flowing of the membrane around the particle which would be energy independent and not be affected by inhibitors of normal endocytosis (Patterson et al, 1979). Thirdly, as has been seen with some viruses, the use of coated pits and receptor-mediated endocytosis is a potential method of chlamydial uptake into the cell (Goldstein et al, 1979).

Ward suggests that receptor-mediated endocytosis is not the mechanism by which chlamydia are taken up into a host cell. This is based upon the evidence that monodansylcadaverine and amantadine (inhibitors of receptor-mediated endocytosis) have no affect on ingestion. This contradicts the data of Soderlund and Kihlstrom (1983), who found that these same agents, while having no effect on attachment, decreased the internalization and intracellular development of chlamydia. However, this effect was negated when the agents were used on confluent monolayers as opposed to subconfluent monolayers. Ward and Murray (1984) were unable to find any clathrin, a major protein of coated pits and vacuoles, in the vacuoles that enclosed chlamydia, even though coated pits and coated vacuoles were seen in the vicinity of the endocytosized chlamydia. While Soderlund and Kihlstrom (1983) did not show chlamydia in coated pits, a recent paper by Hodinka and Wyrick (1986) does show chlamydia within an apparent coated vesicle as well as uncoated vesicles. One consequence of this receptor-mediated endocytosis would be the specific entry into vesicles not destined to fuse with lysosomes, thereby ensuring the survival of the organism. If receptor-mediated endocytosis is not the main mechanism of uptake, what are the alternatives?

Ward and Murray (1984) suggest that the host cytoskeleton is important in uptake of chlamydia. This process would then involve microfilaments and microtubules. Inhibitors of these (cytochalasin B and D for microfilaments and vinblastine, vincristine and colchicine for microtubules) were shown, in general, to inhibit chlamydial uptake. Cytochalasin B was not inhibitory but this is thought to be because it is not effective when the particles are less than 600 nm. Cytochalasin D reduced ingestion by 50%. Vinblastine and vincristine reduced ingestion by about 40%, but colchicine had little effect. The former are thought to disrupt the structure of pre-

formed microtubules while the latter alters the dynamic equilibrium between monomeric and polymeric tubulin which may account for the difference in effect. One interesting observation in support of the "zipper hypothesis" as a mode of chlamydial uptake was made by Ward and Murray (1984). If hyperimmune antisera were added to cell bound chlamydia, their uptake was inhibited. The antibody may have prevented the circumferential binding of the host cell surface to the adherent chlamydia.

The protein calmodulin is thought to be important in both the action of microfilament contraction and receptor-mediated endocytosis. Inhibitors of this protein, such as trifluoperazine, have also been shown to reduce adhesion, uptake of and inclusion development by chlamydia (Murray and Ward, 1984).

4. Reorganization from the EB to the Reticulate Body (RB), Multiplication, Reversion to the EB and Release from the Cell

One area of the study of chlamydia has been observed for a long time, yet it is poorly understood. This is the reorganization of the infectious EB into the non-infectious metabolically active RB. Multiplication takes place by binary fission only in the RB stage followed by conversion back to the EB form and release from the cell. This takes place inside a cytoplasmic inclusion within the host cell and is easy to see with the electron microscope. However, the mechanisms involved remain obscure as the inclusion is surrounded by the host cell.

The ingested membrane bound EB changes from a 300 nm diameter particle to the less dense 900 nm diameter RB. This process starts about four hours after infection of the cell and continues until about 18 hours post-infection when the RB starts to reconvert to form the EB. There is virtually no information on what triggers and sustains any of the processes involved.

What is known are some of the differences between the EB and the RB and some conditions which will inhibit conversion of the EB to the RB.

In order to begin to explain the process, it is necessary to look at some of the major differences between the two life forms of chlamydia. The EB is a 300 nm particle, protein on the outside, condensed DNA on the inside, its sole role being to enter a suitable host cell. It can survive external to the host cell under conditions that the metabolically active RB would not. The tough outer membrane is thought to be highly cross-linked with disulfide bonds which accounts for the rigidity considering its lack of peptidoglycan (Bavoil et al, 1984). In contrast to the EB, although the RB also has a outer membrane, it is very susceptible to osmotic pressure and sonication. In the RB, the DNA is no longer condensed and the RB is very active metabolically.

Competition for amino acids between the chlamydia and the host cell is thought to play a role in development of the parasite. In the laboratory, to enhance chlamydial growth, cycloheximide or emetine are added to shut off the protein synthesis of the host cell and allow the amino acid pool to be used exclusively for the growth of chlamydia. Hatch (1975) found that adding isoleucine alone with no cycloheximide could turn chlamydia from a dormant to a productive state. Recently, Allan and Pearce (1983a) looked at the effect of ommission of a wide variety of amino acids on the multiplication of C.psittaci. They found that deprivation of leucine, phenylalanine or valine completely inhibited inclusion formation while ommission of other amino acids had no effect. In an accompanying paper, these same authors (Allan and Pearce, 1983b) showed that this phenomena is also found among other strains of C.psittaci and in all serovars of C.trachomatis. However, different strains had different amino acid requirements. Stirling

et al (1983) specifically looked at the deprivation of cysteine. This amino acid should be important if disulfide bonding plays a major role in conferring rigidity and stability on the outer membrane. While it did not prevent inclusion formation, ommission of cysteine was found to slow conversion of the RB to the EB. The question was posed as to whether EB envelopes had unique cystein-containing proteins not found in RB. Hatch et al (1984) answered this question by showing that EBs had three extra cysteine-rich outer membrane proteins (MW 62 Kd, 59 Kd and 12 Kd) compared to the RB. Both had almost equal amounts of the major outer membrane protein which also contained cysteine residues. Of interest, the MOMP of RB was much more soluble in SDS alone than EBs which required a reducing agent suggesting that the MOMP was not disulfide linked in the RBs. The rigidity of the EB outer membrane appears to be due to disulfide cross-linking between MOMP and the three other cysteine rich proteins.

Hackstadt et al (1985) looked at the possibility of inducing differentiation using reducing agents such as dithiothreitol and 2-mercaptoethanol (2-me). EBs treated with these agents were shown to increase their rate of glutamate oxidation and their staining characteristics changed to that of RB's although no size increase was observed. The EBs treated with dithiothreitol and 2-ME were also more sensitive to osmotic lysis and had reduced infectivity. Work by Bavoil et al (1984) has shown that the EB outer membrane contains pores whose opening and closing is controlled by a reduction oxidation mechanism. In his model, reduction of the EB outer membrane would open the pores and allow uptake of medium sized molecules (up to 9000 daltons). The membrane would become more flexible and expand to become the RB. As the inclusion matures, the pores in the RB membrane would begin to close and the membrane will become more rigid, forming the EB again.

Recently, Kaul and Wenman (1986) have shown that the addition of cAMP up to 12 hours post-infection will block inclusion development. The total numbers of inclusions are not decreased, but the inclusions failed to mature (reduced size) but only RB were present. These authors identified a cAMP receptor protein (CRP) which was associated with the RB but not the EB. They suggest this CRP may play a role in the regulation of RB to EB conversion.

This work was an extension of the initial observation of Ward and Salari (1982) who found that cAMP at 10^{-3} M reduced inclusion development by two-thirds over control levels. cGMP, on the other hand, was found to increase inclusion numbers by three-fold. These authors suggest, however, that the effect was on the uptake of EBs into the cell rather than enhanced inclusion development. It was suggested that the cAMP might increase membrane fluidity while the effect of the cGMP could be due to its ability to inhibit prostaglandin biosynthesis.

5. Immune Response to Chlamydial Infection

It has been amply demonstrated that chlamydial infections elicit a strong and specific immune response, both humoral and cell-mediated. Within the scope of this thesis, the work on humoral immunity will be emphasized with some reference to the literature as concerns cell-mediated immunity.

In general, the literature tends to look at the immune response as a result of chlamydial infection of the eye (trachoma) or the genital tract. An excellent review by Kunimoto and Brunham (1985) summarizes the main finding in chlamydial infections. C.trachomatis is trophic for columnar and transitional epithelial cells on the mucosal surface. The histo-

pathology of trachoma shows chronic inflammation in all tissues, both mucous and submucous. In the case of trachoma, there appears to be eventual proliferation of scar tissue resulting in scar formation. The authors suggest similar histopathological changes take place in C.trachomatis infection of the cervix. Such infection processes in the fallopian tube with scar formation could lead to ectopic pregnancy or tubal infertility. An infection of the guinea pig eye caused by a strain of C.psittaci produces guinea pig inclusion conjunctivitis (GPIC) (Monnickendam et al, 1980; Murray et al, 1973). This animal model has been used to study both humoral and cell-mediated immunity to chlamydial infections.

It was shown by Blyth et al (1962) that antisera raised against chlamydia could neutralize infection in vitro. Antibodies produced by natural chlamydial infection (trachoma) have also been able to neutralize infection in vitro (Howard, 1975; Barenfanger and MacDonald, 1974). The role of antibody in humans is not known. If antibody is found in the tear fluid, it generally indicates an active infection is present (Treharne et al, 1978). In the GPIC model, immunity to conjunctival infection was found only with animals having secretory IgA in their tear fluid (Murray et al, 1973). Specific serum antibodies did not appear to prevent infection. Serum IgA and IgG fractions were protective when used to opsonize the mouse C.trachomatis strain in a chlamydia pneumonia model (Williams et al, 1984). Overall, the antibody reaction in trachoma tends to be type specific (Nichols et al, 1973), suggesting the neutralizing antibodies are being made against the MOMP as, to date, this is the only protein of chlamydia known to carry a type specific determinant. Recent data by Ward et al (1986) characterized a number of trachoma sera by MIF as being specific for serotype A or B. However, when used in immunoblots, the sera did not

show particularly strong responses to their homologous MOMP. A number of other polypeptides were also recognized by this sera and a special note was made to strong reaction with a polypeptide of about 63 Kd MW in a number of sera. In summary, for trachoma infections, humoral immunity may play a role in limiting these infections, but it does not appear to prevent the initiation of infection.

The same generalizations follow for chlamydial infections of the genital tract. An active infection is indicated if local antibodies are demonstrated than if just serum antibodies to chlamydia are found (McComb et al, 1979). Brunham et al (1983) found a similar correlation with isolation rates and the presence of secretory IgA. However, it was interesting that the higher the antibody titre, the lower the number of organisms isolated. This relationship could account for the results seen by McComb et al (1979) in which women with high antibody levels in their cervical secretions and serum had no organisms isolated. High titres of chlamydial antibody have been reported from infertile women with damaged fallopian tubes (Conway et al, 1984). While to date no one has used genital secretions to neutralize infection, two studies in animals suggest that humoral immunity is important in the recovery from and prevention of chlamydial genital infection (Rank and Barron, 1983; Rank et al, 1979). When the humoral immune system was suppressed with high doses of cyclophosphamide in guinea pig infected with GPIC intravaginally, there was a prolonged infection compared to the controls. Similar results were seen when guinea pigs cured of an initial infection were rechallenged. Only those animals with suppressed humoral immunity became infected.

Some specific analysis of the antigens recognized during genital infection of women has been done (Newhall et al, 1982). There were three

main antigens which reacted with antibody. These were the MOMP and two proteins of 60 Kd and 62 Kd. Ward et al (1986), using sera from LGV patients, found reaction to antigens of 19 Kd, 29 Kd, 41 Kd, 58 Kd, 63 Kd and 65 Kd. Unlike Newhall, who found some weak responses to MOMP, Ward showed that their major antibody response was against the MOMP. It must be remembered that LGV disease is much more severe than the initial genital non-LGV infection seen in Newhall's study, which could cause the antibody responses to be quite different.

A number of authors have tried to identify the point of neutralization by antibody in the chlamydial life cycle and the antigens involved. Friis (1972) suggested that antisera, while allowing uptake of the EB into the cell, would prevent inhibition of phagolysosomal fusion resulting in no infection. Ward and Murray (1984) used immune sera to obtain results suggesting that uptake of the organism into the host cell is prevented by antibodies. Caldwell and Perry (1982) used rabbit antisera prepared against purified L2 MOMP to neutralize infection of HeLa cells with serovar L2. Attachment and internalization were not inhibited. The suggestion was that cross-linking of the MOMP molecules was required for neutralization. This might prevent the reorganization from the EB to the RB. While not required, complement was found to enhance neutralization. Lucero and Kuo (1985), using MAbs against the MOMP, found that neutralization was complement dependent. Of interest, not all MAbs to MOMP tested were neutralizing. Five of seven serovar specific and two of five subspecies specific MAbs could neutralize while no species or genus specific MAb could neutralize. Recently, Wenman and Mueser (1986) used immune sera against an 18 Kd protein to neutralize infection. This protein is thought to be the ligand which binds to HeLa cell receptors. Antisera should prevent attachment although this was not investigated.

In summary, it appears that antibody to chlamydia, raised in animals or from naturally infected humans, can neutralize infection in vitro. The animal models also show prevention of infection if chlamydia specific antibodies are present. However, there does not appear to be immunity to repeated infections in humans as seen by the ongoing infection of trachoma. The majority of neutralizing antibody seems to be directed against the MOMP.

6. Antigens of Chlamydia

The study of chlamydial proteins, as with those of other organisms, gained momentum with the use of polyacrylamide gel electrophoresis, which allowed visualization of individual proteins and gave an estimate of their molecular weight. One of the first applications of this technique to chlamydia was by Becker et al (1971). The tube gel showed 38 distinct proteins with the majority of ^{14}C-amino acid label being found in one band about half-way down the gel. This is probably the first visualization of the chlamydial MOMP. Tamura et al (1974) was able to visualize 9 separate proteins in cell wall preparations of EBs and RBs. The cell walls had been extracted using sonication, trypsin digestion and SDS treatment (Tamura and Manire, 1967; Manire and Tamura, 1967). Again, the most prominent band was one around 40 Kd. There were no major differences in protein profiles between EB and RB preparations.

Caldwell et al (1975a) used two-dimensional immunoelectrophoresis to compare the antigens of chlamydia. They found 19 and 16 distinct antigens from C.trachomatis L2 and C.psittaci Cal-10 respectively, but only one was common between the two. This antigen was in the supernatant fraction of EBs solubilized with triton X-100. In a companion paper, Caldwell et al (1975b) described a C.trachomatis specific antigen which was recognized by

15 of 18 sera from individuals infected with C.trachomatis. This antigen was later purified and shown to have a MW of 155 Kd (Caldwell and Kuo, 1977a). When used in a counter immunoelectrophoresis assay, 43 of 45 LGV patient sera possessed antibody against this antigen (Caldwell and Kuo, 1977b). However, the 155 Kd protein was a very minor component in the gel profile in comparison to the rest of the protein complement, an observation which Caldwell appeared to ignore at the time. Sacks and MacDonald (1979) used nonidet-P40 to solubilize serovar A EBs and described the isolation of a type specific antigen of MW 27 kd. No gels were shown so it is hard to judge the information in this paper. In summary, to this point, only two proteins of MW 27 Kd and 155 Kd have been specifically described within the 38 proteins seen in whole EBs overall and the 9 proteins seen in cell wall preparations. No functions have been ascribed to the proteins except that there was hemagglutinin activity in EB cell wall preparations (Tamura and Manire, 1974) and that antibody to the 155 Kd protein was present in LGV patients. However, this work set the stage for the relative explosion of information on chlamydial antigens which occurred in the 1980's.

a) The Chlamydial Major Outer Membrane Protein (MOMP). Extensive study of the MOMP began in 1981 in a rather interesting way. While two papers (Salari and Ward, 1981; Hatch et al, 1981) described the initial recognition of this protein in both C.trachomatis and C.psittaci, Caldwell et al (1981) reported its complete purification and preliminary characterization. The MOMP was seen to have a variable MW (38 Kd to 42 Kd) depending upon the serovar and was surface exposed. It was noted that the MOMP was found in both EB and RB, making up about 25% of the total protein of purified EB (Hatch et al, 1981). It was the classic paper by Caldwell et al (1981), however, that really opened up the research on chlamydial antigens. In their attempts to purify the MOMP, it was seen that EBs treated

with 2% sarkosyl would leave behind an intact outer membrane that was heavily enriched in MOMP. The MOMP, which makes up 60% of the total protein found in this complex, could be completely extracted using 2% SDS at 60°C. Knowing that chlamydiae lack detectable amounts of peptidoglycan, it was suggested that the MOMP, in association with other membrane proteins, could provide the structural stability of the EB outer membrane. Just what kind of interaction held the complex together was not known.

Hatch et al (1981) first suggested that disulfide bonding might hold the outer membrane together and a number of authors have since confirmed this idea. MOMP has been seen to exist in a number of multimeric forms such as dimers and trimers when separated by PAG under non-reducing conditions (Newhall and Jones, 1983). While seen with EBs, these forms were not seen with RBs, suggesting disulfide bonds may be important in conversion from one form of chlamydia to another. Caldwell et al (1981) had suggested that MOMP was insoluble in a variety of detergents except SDS, but Bavoil et al (1984) and Nurminen et al (1984) showed that it was soluble in detergents as long as a reducing agent was present. Hackstadt et al (1985) treated whole EBs with 10 mM DTT and found that they took on some of the characteristics of RBs such as enhanced rates of glutamate oxidation, reduced infectivity and decreased osmotic stability. The MOMP was the protein most affected. Again, dimers and trimers of MOMP were seen in the absence of a reducing agent. The MOMP of RBs was found to be more highly labelled with ^{14}C-iodoacetamide than the MOMP of EBs, suggesting that there are more free sulfhydral groups on the RB than on the EB. It was felt that reduction of the EB outer membrane must take place in order for conversion to the RB to occur. Overall, MOMP appears to be the major structural protein of the chlamydial outer membrane, cross-linked by disulfide bonds. Bavoil et al (1984) have also suggested it may function as a porin, opening

and closing under reducing and non-reducing conditions to allow the passage of nutrients. While the MOMPs of the various serovars may function in the same way, how closely are they related?

Caldwell and Judd (1982) and Caldwell and Schachter (1982) used one and two-dimensional peptide mapping of purified MOMP to show that while different serovars had regions of homology, there were significant areas of heterogeneity. Polyclonal sera raised against pure MOMP from different serovars showed the highest titres with the homologous serovar, but there were cross-reactions with heterologous serovars. This showed that a particular MOMP had a number of different antigenic sites which are suface exposed, some of which are shared with other serovars.

While peptide mapping showed rough similarities between different MOMPs, it was through the use of monoclonal antibodies (MAb) that definite comparisons between MOMPs could be made. Because MAb are specific for a given epitope, they can be used to give unequivocal results as to just how similar or dissimilar two different MOMPs may be. Stephens et al (1982) started with the production of 13 MAbs against the MOMPs from the serovars B, C and L2. He found that these MAbs fell into three categories. Some were type specific, meaning the epitope was limited to one serovar only, while others were subspecies specific, reacting with two or more different serovars and still others were species specific, reacting with all serovars but not with C.psittaci. The patterns of reaction also correlated well with the results seen using the MIF assay and polyclonal sera raised against whole EBs (Wang and Grayston, 1977). Differentiation of serovars using the MIF assay is based upon reaction of antibodies with the MOMP. Other authors have produced MAb to chlamydial MOMP, such that type specific MAb to all the serovars have been made except for Ba, J and L_3. The most

interesting MOMP MAbs are the subspecies group as they show the interrelationship between the various serovars and are listed in Table 2.

While the earlier work of Wang and Grayston (1977) with polyclonal sera established rough antigenic boundaries for the various serovars, the use of MAbs allows precise definition. They have supported the initial findings and have gone on to identify new relationships. For example, the C complex was thought not to have epitopes in common with L3, but at least one MAb reacts with serovars C and L3 and within the C complex, two MAbs react with serovars J, G and L3 which initially were thought to be antigenically unrelated. All of these different MAbs also show the large number of different epitopes on the MOMP. Serovars B and Ba each have 10 and 11 different epitopes, respectively, which cross-react with other serovars. These MAbs will be important in the epitope mapping of the immunogenic portions of the MOMP which will hopefully lead to the identification of neutralizing epitopes with the potential for use as a peptide vaccine.

One benefit of the production of MAbs to the MOMP has been the production of tests which allow rapid, sensitive and specific diagnosis of chlamydial infection in humans. The MAbs have also been used to try and prevent chlamydial infections in vitro. Theoretically, a strong antibody response against the main component of the outer membrane should be able to stop infection. It is therefore interesting that while some MAb to MOMP can neutralize infection, the majority of them are unable to do so (Lucero and Kuo, 1971; Peeling et al, 1984). Whether certain areas of the MOMP molecule are more susceptible to neutralization or whether it is the action of a specific type of Ig is not known at this time. The fact that antibody reactive with the MOMP can neutralize infection has spurred interest in it as a component of a protective vaccine. Certain epitopes on MOMP are

TABLE 2. Micro-IF reaction patterns of subspecies MOMP monocloncal antibodies (MAbs) reported in the literature to EBs from 15 chlamydial serovars.

C[a]	J	H	A	I	G	L3	F	K	B	Ba	E	L1	D	L2	Ref.
									+	+	+	+	+	+	1,2,3,4
						+		+	+	+				+	1
									+	+					2
									+	+			+		2
					+	+	+	+	+	+	+	+	+	+	4
					+	+	+		+	+	+	+	+	+	2
					+		+		+	+	+	+	+	+	4
+	+	+	+	+		+		+							4
							+		+	+		+	+		4
									+	+				+	4,5
							+					+	+		4
					+	+									4
+	+														3,4
					+		+								4
+						+									4
+	+	+	+	+											3,6
			+	+											3
	+				+	+									3
					+		+				+			+	3
							+	+		+				+	3
					+		+						+		3
											+	+			3
											+		+	+	3
								+	+	+	+		+	+	3
	+	+			+	+									5

Ref.
1. Stephens et al (1982)
2. Zhang et al (1987)
3. Barnes et al (1985)
4. Batteiger et al (1986)
5. Wang et al (1985)
6. Clark et al (1982)

a Chlamydial serovars.

species specific or common to all serovars. These would be the regions of MOMP valuable to use as a vaccine, however, no MAb to the species specific epitope causes neutralization. The specific epitopes which react with neutralizing antibody of each serovar will have to be examined to determine what makes them effective. This involves knowing their amino acid sequences and eventually the DNA sequences coding for the various MOMPs.

To date, three groups have had success with studies on the expression and sequencing of the MOMP gene. Allan et al (1984) first cloned DNA from serovar L1 into a phage vector with expression in E.coli. A 40 Kd polypeptide was produced which was reactive with immune sera and gave the same peptide map as chlamydial MOMP. While this process does not allow purification of MOMP on a large scale, they did show the gene could be cloned. Nano et al (1985) took a different approach to their search for the MOMP gene. While Allan et al (1984) had relied on the shotgun method of DNA cloning, Nano synthesized a MOMP gene probe based on the sequence of the first 25 amino acids of MOMP from serovar L2. They had partial success in that truncated portions of the MOMP were produced which would react with some MOMP MAbs but not all. Stephens et al in two papers (1985, 1986) has completely sequenced the gene coding for the L2 MOMP. This is a major breakthrough as it will allow indepth analysis of the different regions of the MOMP. Also of interest was that the gene codes for nine cysteine residues which would account for the high degree of inter- and intra-protein disulfide bonding thought to occur among the MOMP and other outer membrane proteins. Again, the MOMP gene was not cloned into a vector suitable for production of the MOMP in substantial amounts.

b) Lipopolysaccharide (LPS). Lipopolysaccharide is a major component of the outer membrane of gram-negative bacteria. This molecule, which is also found in Chlamydiae, has been extensively studied and has been found to be

made up of three distinct antigenic regions (Hitchcock et al, 1986). The general structure of LPS is as follows. One section is the O-specific antigen which is made up of a long chain polysaccharide of repeating units of one to seven sugars. This is linked to the oligosaccharide core which has up to 10 monosaccharides including the sugar KDO (3-keto-2-deoxyoctonate), which is, in turn, linked to the third component of LPS, the lipid A. The lipid A is made up of a lipid backbone of diglucosamine in B 1-6 linkage with various fatty acids in an amino or hydroxyl linkage. Because KDO is a component of most gram-negative bacterial LPS, its presence in chlamydia might indicate chlamydial LPS. Dhir et al (1972) found this sugar in what was termed a genus specific antigen in both C.trachomatis and C.psittaci. Nurminen et al (1983) were able to isolate LPS-like material from serovar L2 and compared it to the LPS of a rough mutant (contains only the lipid A core and KDO oligosaccharide and lacks the O polysaccharides) of Salmonella typhimurium. The two structures were found to be immunologically cross-reactive and functionally related, suggesting that chlamydia has a rough type LPS with no long polysaccharide chains (Nurminen et al, 1984). In a follow-up paper, Nurminen et al (1985) chemically characterized L2 LPS as having "typical lipopolysaccharide components". These included D-glucosamine, long-chain 3-hydroxy fatty acids, 2-keto-3-deoxyoctanoic acid and phosphate.

The LPS of chlamydia has been studied using both polyclonal antisera and MAb. Nurminen et al (1983, 1984) in two papers described the immunological cross-reaction between the LPS of chlamydia and salmonella. Brade et al (1985) showed that there were at least two antigenic domains on the chlamydial LPS. One which cross-reacts with enterobacterial rough LPS and the other which was restricted to the genus chlamydia. Using both MAb and polyclonal antisera, Caldwell and Hitchcock (1984) suggested there were

three antigenic domains on the chlamydial LPS, two of which are shared with other gram-negative bacteria and one which is specific for chlamydia. Thornley et al (1985) produced two types of MAbs. One set was specific for the chlamydial LPS and the other for the domain common to chlamydia and salmonella. All of the information suggests at least two, if not three, main antigenic domains on the chlamydial LPS.

Recently, Watkins et al (1987) has shown that the chlamydial LPS is the hemagglutinin previously described by other authors. She suggests that the lipid A moiety of the LPS binds to the erythrocyte membrane which causes a change in the surface charge such that agglutination occurs. Interestingly, only mouse and rabbit erythrocytes were agglutinated while those of human and guinea pig did not agglutinate even though they bound an equivalent amount of LPS.

Nano and Caldwell (1985) were able to clone part of the chlamydial genome into an E.coli strain which allowed expression of the chlamydial genus specific epitope. Using the recombinant E.coli in cell association experiments with HeLa cells, the authors showed two to five times greater association than the parent strain, suggesting the chlamydial LPS may play a part in chlmaydial-host cell interaction, however, to date, none of the LPS specific MAb or polyclonal sera have been able to neutralize infection.

c) Non-MOMP; Non-LPS Antigens. While the MOMP and LPS are the most prominent antigens of the outer membrane, it must be remembered that other proteins are present. Humans naturally infected with chlamydia and laboratory animals immunized with whole EBs make antibodies to antigens besides MOMP and LPS. Radio-iodination experiments with whole EBs have also indicated a number of other major proteins as being part of the outer membrane. A number of groups are now turning their interest to these antigens.

Two proteins previously described are 18 Kd and 32 Kd antigens which may be the molecules which attach to the eucaryotic cells (Hackstadt, 1986a; Wenman and Meuser, 1986). However, there was no solid evidence offered to suggest that these proteins were surface exposed as part of the outer membrane.

Interest has focused on outer membrane proteins which may be linked to the MOMP by disulfide bonds. Newhall and Jones (1983) identified proteins at 60 Kd and 15 Kd, which they felt might be linked to the MOMP. Proteins with similar MW were identified as being cysteine enriched in both C.psittaci and C.trachomatis (Hatch et al, 1984; Batteiger et al, 1985). In a recent paper, Newhall (1987) suggests that as the chlamydial inclusion develops, there is progressive cross-linking of the MOMP with two 60 Kd proteins and a 12.5 Kd protein (which may be the 15 Kd protein). How this cross-linking is controlled is not known, although Newhall suggests some sort of disulfide interchange enzyme may play a role. Another protein which has been cloned is a 75 Kd molecule. Kaul and Wenman (1985) suggest that this protein is part of the outer membrane but ascribe no function to it.

Thus, the available literature suggests that the complicated life cycle exploited by chlamydiae is, in part, determined by outer membrane macromolecules. The molecular basis for eucaryotic cell infection is, at present, unknown.

We therefore undertook a detailed comprehensive study of chlamydial antigens in order to identify those which are common among chlamydiae and which are located in the chlamydial outer membrane. Our experimental hypothesis was that outer membrane antigens common among the genus chlamydiae are important in the pathogenesis of infection and are possible immunoprotective antigens.

MATERIALS AND METHODS

1. List of Chlamydial Strains

The C.trachomatis serovars used were A (G17/OT), B (TW5/OT), C (TW3/OT), D (UW3/Cx), E (UW5/Cx), F (UW6/UR), G (UW57/Cx), H (UW4/Cx), I (UW12/UR), J (UW36/Cx), K (UW31/Cx) and L2 (434/BU) (courtesy of C-C Kuo, University of Washington, Seattle). C.trachomatis mouse pneumonitis (MoPn) (Weiss strain) and C.psittaci meningopneumonitis (CpMn) were obtained from J. Schachter (University of California, San Francisco) and W. Wenman (University of Alberta, Edmonton), respectively.

2. Growth of Chlamydial Strains and Purification of EBs

This process involves a number of steps which have been standardized by our laboratory and others working with chlamydia. The four individual procedures are outlined below with the complete details being taken in better part from a manual prepared in our laboratory by R. Peeling and J. McDowell.

a) Propagation of HeLa 229 Cell Cultures.

b) Growing Chlamydia.

c) Preparation of Chlamydia to be Used as Innoculum.

d) Preparation of Purified EBs.

a) Propagation of HeLa 229 Cell Cultures. HeLa 229 cells (American Type Culture Collection, Baltimore, Maryland) were grown in Eagle's minimum essential medium (MEM) with 10% fetal calf serum (GIBCO) (Here after known as MEM).

1. Discard medium of an established cell culture flask (Nunc, 175 cm^2, GIBCO).
2. Rinse the cells with GKNP 1x (Appendix A), about 5 ml.

3. Rinse with 3 ml of 0.05% trypsin solution (Appendix A).

4. Add just enough trypsin to cover monolayer (1-2 mls for 175 cm^2 flask) and incubate at 37°C for a few minutes.

5. When the cell layer starts to detach, add 5 ml of MEM and pipette vigorously to disperse the cells.

6. If a confluent monolayer is required in 24 hrs, split the cell suspension from 1 into 2 flasks. Otherwise, the suspension can be used to seed 3 flasks. For maintenance, discard 2/3 of cell suspension and put remaining 1/3 back into original flask.

7. Place about 50-75 ml of MEM in each flask and incubate at 37°C.

b) Growing Chlamydia

i) Growing Chlamydia trachomatis Serovars A-K + Mouse Pneumonitis

1. Prepare a confluent 24 hr monolayer of HeLa 229 cells in a 175 cm^2 flask.

2. Discard MEM and add 3 ml of Hank's BBS (Appendix A) containing 30 ug/ml of DEAE-dextran (Pharmacia).

3. Let stand at room temperature (R.T.) for 20 min.

4. Remove DEAE-dextran and add 1 ml of chlamydia stock.

5. Let stand at R.T. for 2 hours.

6. Discard inoculum, rinse monolayer with HBSS and add 50-75 ml of MEM with cycloheximide (1 ug/ml).

7. Incubate for 60-72 hours at 35°C*.

ii) Growing LGV Serovars

1. Prepare a confluent 24 hr monolayer of HeLa 229 cells in a 175 cm^2 flask.

2. Discard MEM and rinse monolayer with HBSS.

3. Discard HBSS and add 1 ml of LGV stock.

4. Let stand at R.T. for 2 hours.

5. Discard inoculum, rinse monolayer with HBSS, and add 50-75 ml of MEM with cycloheximide.

6. Incubate for 36-48 hours at 37°C*.

* It is our experience that a heavy inoculum, while not destroying the monolayer, will cause the inclusions to develop more quickly such that 72 hr and 48 hr are not hard and fast time periods.

iii) Growing C.psittaci

1. Prepare a confluent 24 hr monolayer of HeLa 229 cells in 175 cm^2 flask.

2. Discard MEM and rinse monolayer with HBSS.

3. Discard HBSS and add 1 ml of C.psittaci stock.

4. Let stand at R.T. for 2 hours.

5. Discard inoculum, rinse monolayer with HBSS and add 50-75 ml of MEM with cycloheximide (1 ug/ml).

6. Incubate at 37°C for 24 hours.

7. Discard medium, add 10 ml of fresh MEM with cycloheximide and incubate another 24 hrs at 37°C before harvesting.

c) Preparation of Chlamydia to be Used as Inoculum

1. Discard medium from cell culture (except for C.psittaci where the 10 ml of medium is retained for harvesting).

2. Add 10 ml of cold HBSS and approx. 30 glass beads (2 mm) to flask.

3. Roll cells gently off culture flask.

4. Transfer cell suspension to a 30 ml polycarbonate centrifuge tube kept on ice.

5. Rinse flask with 10 ml of HBSS and add to centrifuge tube.

6. Sonicate cell suspension for 35 seconds at a setting of 3 (Microprobe, Branson Sonifier, Danbury, Connecticut).
7. Centrifuge at 500 x g for 15 min at 4°C (Sorvall, SS-34 rotor).
8. Discard pellet and centrifuge supernatant containing chlamydia at 30,000 x g for 30 min (Sorval, SS-34 rotor).
9. Resuspend pellet in 6-8 ml of SPG (Appendix A) using a 10 ml syringe and blunted needle (20 guage spinal tap, Becton-Dickenson).
10. Culture stock for sterility.
11. Divide into 1 ml aliquots and store at -70°C (each 1 ml aliquot should give 95-100% infectivity in a 175 cm^2 flask).

d) Preparation of Purified Chlamydial EBs

1. Discard medium from cell culture (except for C.psittaci where the 10 ml of medium is retained for harvesting.
2. Add 10 ml of cold HBSS and approx. 30 glass beads (2 mm) to flask.
3. Roll cells gently off culture flask.
4. Transfer cell suspension to a 30 ml polycarbonate centrifuge tube kept on ice.
5. Rinse flask with 10 ml of HBSS and add to centrifuge tube.
6. Sonicate cell suspension as before.
7. Centrifuge at 500 x g for 10 min at 4°C.
8. Discard pellet and layer supernatant over 8 ml of 35% renograffin (Renograffin 76, Squibb, Canada) in HEPES buffer (.01 M HEPES/.15 M NaCl, pH 7.2) in a 50 ml ultra-clear tube (Beckman Instruments) (max. 4 x 175 cm^2 flasks per 8 ml cushion).
9. Centrifuge at 43,000 x g (Beckman ultracentrifuge 16,000 rpm) for 60 min at 4°C in a SW27 rotor.

10. Resuspend pellet in 10 ml of SPG.

11. Layer over a discontinuous renograffin gradient containing:

13 ml 40% renograffin in HEPES (top)

8 ml 44% " (middle)

5 ml 52% " (bottom)

(max. 2-3 flasks/gradient)

12. Centrifuge at 17,000 rpm for 90 min at 4°C in SW27 rotor.

13. Collect EBs at 44-52% interface.

14. Dilute with 3 volumes SPG or Hepes.

15. Centrifuge at 30,000 x g (Sorval 16,000 rpm) for 30 min at 4°C.

16. Repeat wash.

17. Resuspend EBs in 1 ml SPG per flask.

3. Protein Determination

Two different methods were used for estimation of the protein content of an unknown sample. One of these was based upon an assay designed by Bradford (1976). It was used for proteins which were in soluble form. The unknown would be diluted to give a number of samples with a protein content between 1 and 25 ug/ml. 800 ul of this would be added to 200 ul of Dye Reagent Concentrate (Bio-Rad), vortexed and allowed to stand at least 5 minutes. The OD (595 nm) would be used against a reagent blank with no protein. A standard curve would be produced in the same way using bovine serum albumin.

The Bradford method is not compatible with a number of compounds, including SDS, which is necessary for the solubilization of chlamydial EBs. A second assay system was used based upon the quantitative binding of amido black to protein immobilized on a nitrocellulose membrane (Schaffner and Weissmann, 1973). In this procedure, the protein sample would be

diluted in 220 ul of dH_2O. To this, 30 ul of 1 M TRIS/2% SDS pH 7.5 would be added, vortexed and then 50 ul of 90% TCA was added, vortexed and allowed to sit at least 2 minutes. This solution was filtered through a .45 um nitrocellulose membrane in a Bio-Dot (Bio-Rad) apparatus. The tube was rinsed with .4 ml of 6% TCA and filtered. The filter was stained in .25% naphthal blue black in 50% methanol/10% acetic acid for 15 minutes, then rinsed with water for 45 seconds. The filter was destained with 3 rinses of 90% methanol/2% acetic acid. After blotting dry, the spots were cut out and the dye eluted with 1 ml of 50% ethanol/25mM NaOH/50 um EDTA for 5 minutes. The absorbance was read at 620 nm with BSA again being used to produce a standard curve.

4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gels were used to separate proteins or protein fragments according to molecular weight. The discontinuous buffer system of Laemmli (1970) was used with all reagents being of electrophoresis purity grade (Bio-Rad). Gel concentrations ranged from 10-18% and two gel apparatuses were employed. The first was a Protean Dual Slab Gel Unit (Bio-Rad) with gel dimensions of 16 cm x 14 cm x .75 cm. The second unit was a Mini Protean II (Bio-Rad) with gel dimensions of 8 cm x 10 cm x .75 cm. The first would take about 4 hours to run and was used for fine resolution of protein bands while the second had a running time of about 30 minutes and was used more for quick analysis of protein content of various samples.

On a given run, 2-5 ug protein would be loaded per well after solubilization (Appendix A). The gels were run in .025 M TRIS/.192 glycine/.1% SDS at 20 mAmps per gel for the large apparatus and at 200 volts for the small unit, both until the tracking dye reached the bottom of the gel.

Estimation of molecular weights was done using the molecular weight standards lysozyme (14,400), soy bean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200) and phosphorylase B (92,500) supplied by BioRad. The gels were then either silver stained (see below) to visualize protein bands or electro blotted (see below) to transfer the protein bands to a nitrocellulose membrane.

5. Silver Staining of Polyacrylamide Gels

The procedure used to stain the gels for visualization of the protein bands was after the method of Morrissey (1981). Special care was taken to prevent dirt or grease from touching the gels such that glass staining dishes were prewashed in detergent and the gels were handled only with disposable latex gloves which were washed with soap to remove any powder.

Following a PAGE run, the gel would be immersed in 100 ml of 50% methanol/10% acetic acid/40% dH_2O for 30 minutes with gentle shaking. This would be poured off and 100 ml of 5% methanol/7% acetic acid/88% dH_2O added for another 30 minutes with shaking. After this solution was poured off, 100 ml of 10% glutaraldehyde was added for 30 minutes, then the gel was washed with three changes of dH_2O and left to sit overnight in 300 ml dH_2O. The dH_2O would be poured off and 200 ml of a dithiothreitol solution (5 ug/ml in dH_2O) was added for 30 minutes, poured off and 200 ml of a silver nitrate solution (1 mg/ml in dH_2O) added with shaking for 30 minutes. This was poured off and the gel washed three times to remove unbound silver nitrate. The gel was developed with 250 ml of a 3% sodium carbonate solution in dH_2O with 125 ul 37% formaldehyde. 50 ml of the developer was added to rinse the gel, poured off and the remainder added until the desired level of staining was attained. The developer would be poured off and the gel rinsed extensively with dH^2O. The gel was either photographed or dried for a permanent record.

6. Western Blotting

This procedure involves the transfer of proteins separated by SDS-PAGE onto a nitrocellulose membrane (NCM) allowing them to be reacted with immune sera or MAbs which is not possible when they are in a gel matrix. A variation on the original method of Towbin et al (1979) was used in our laboratory.

Following the PAGE run, the gel would be immersed in transfer buffer (25 mM TRIS/192 mM glycine/20% methanol, pH 8.3) for 1 hour with shaking to remove excess SDS. The NCM (BioRad, .45 um) was cut to fit the gel and presoaked in transfer buffer for 30 minutes. A TRANS BLOT apparatus (BIO-RAD) was used for the electroblotting.

The NCM was placed on wetted 3 MM chromatography paper and the gel piece laid on top of it, making sure to exclude any air bubbles. Another piece of wetted 3 MM paper was laid on top and this sandwich placed in between two pieces of Scotch brite pads and placed in a TRANS BLOT holder such that the gel side was towards the cathode. The apparatus was filled to the top with transfer buffer. It was found that best transfer of protein to the NCM was obtained with overnight blotting using .15 Amps followed by an increase to .20 Amps for 1 hour the next morning.

The NCM with bound protein was immersed in a blocking buffer (4% BSA or 10% horse serum in .01 M TRIS/.15 M NaCl, pH 8.2) for 1 hour at 37°C with shaking. This was done to prevent non-specific binding of immunoglobulin to areas on the NCM where no protein had been attached. The antibody was then added in a volume of 5-10 ml. It was diluted in the wash buffer (.01 M TRIS/.15 M NaCl/.2% TWEEN 20, pH 8.2) with .1% BSA. Tween 20 is a detergent used to prevent non-specific sticking of the antibody to the NCM (Batteiger et al, 1982). The amount of antibody added to the NCM depends upon the titre of the immune sera or MAb. The NCM was sealed in a

bag with the diluted antibody and incubated at 37°C for 1-2 hours. It was then washed three times over 30 minutes in wash buffer and then the second antibody is added. This antibody was selected such that it will bind to the first antibody. For example, the second antibody might be rabbit antisera specific for mouse IgG or goat antisera specific for human IgA. To be detected, the second antibody has some type of label, usually an enzyme such as alkaline phosphatase or horse radish peroxide. In our laboratory, the second antibody was tagged with horse radish peroxidase (HRP) and was used at a dilution of 1:3000 in wash buffer plus .1% BSA. Again, 5-10 ml was used per NCM in bags. This incubation step was carried out at 37°C for 1-2 hours followed by three washes in wash buffer.

The blots were developed with diaminobenzidine [Sigma, .5 mg/ml in .01 M Na phosphate buffer/.15 M NaCl, pH 7.2 (PBS)]. Normally, 100 ml of developer was used to which 100 ul 3% H_2O_2 would be added. Full development of the brown colouration would take about 10 minutes and then the NCM would be washed three times with dH_2O and blotted dry.

The different dilutions of immune sera and specific MAbs, along with the various second antibodies used, will be discussed in the relevant section rather than presenting the information in this section.

7. Immunoblotting of Human Sera

Approximately 5 ug of whole EBs (serovars L2, D, I, J) were solubilized and run on 10% PAG and then electroblotted to NCM. The blots were blocked with 4% BSA for 1 hour and then immune sera was added. The sera were obtained from women who were infertile due to scarring of the fallopian tubes and had antibodies to chlamydia as determined by MIF. A control group consisted of women who were pregnant but also had antibodies to chlamydia as determined by MIF.

The first antibody (human sera) was added to the blots at a dilution of 1/500 when IgG was being tested and 1/100 for both IgA and IgM. The second antibody was used at 1/3000 dilution and was rabbit immunoglobulin (HRP labelled) against human IgG, human IgA, or human IgM (DAKO).

8. Production of Monoclonal Antibodies to C.trachomatis Antigens

In general, the method of Stephens et al (1982) was followed with some alterations. We undertook three separate fusion experiments using the serovars L2, J and G as immunogens. The procedures in each case were essentially the same such that the J fusion will be described in detail as an example of how the MAbs were produced. The process is made up of three individual steps. The first is the immunization of the mice, followed by fusion of the immune spleen cells with a myeloma cell line and lastly, detection of the hybridoma clones producing the desired antibody.

The immunization schedule consisted of four separate injections, each one week apart, of 100 ug of whole chlamydial EBs. The mice used were female Balb/C 4-6 weeks old (obtained from the University of Manitoba Animal Care Services). The first injection was given intraperitoneally (.25 ml EBs in SPG + .25 ml Freunds complete adjuvant) at day 0. On day 7, the same amount, except using Freunds incomplete adjunvant, was given subcutaneously. On day 14, .5 ml EBs in saline were injected intravenously through a tail vein. The booster dose of .5 ml EBs in saline was given on day 21 and the spleens were excised on day 24 or 25. Just before removal of the spleens, about 1 ml of blood was collected, spun down and the serum saved for analysis of the antibody response to chlamydial antigens.

Once the spleens have been removed, the process of fusing the spleen cells with the myeloma cell line, followed by plating in microtitre plates, takes about 5 hours. In the case of the J fusion, the spleens from two

immunized mice were excised and placed in 10 ml of RPMI 1640 (GIBCO) media on ice. The spleens were washed once and then put into a sterile petri dish and cut into small pieces. Complete mincing of the spleen into single cells was carried out by mashing the pieces with the end of a pasteur pipette. This was done over a period of 15 minutes with five washings of the cells into a 15 ml centrifuge tube. When the spleen was completely broken up, about 10-12 ml would have been collected. The tube was inverted and 2 minutes allowed for any large pieces to settle out. The supernatant was transferred to another 15 ml tube. This was centrifuged at 160 x g for 5 minutes at R.T. The pellet was washed once in 10 ml RPMI 1640, then resuspended in 10 ml .85% ammonium chloride in dH_2O for 5 minutes to lyse any red blood cells. This was centrifuged 160 x g for 5 minutes and the pellet was washed once in 10 ml RPMI 1640 and resuspended in a final volume of 10 ml RPMI 1640. The cells were counted in a haemocytometer. 50 ul of the cell suspension was added to .90 ml PBS and 50 ul 1% trypan blue. The counts of the J fusion from the two spleens combined were 5.9×10^7 cells/ml with a total of 10 ml.

The mouse myeloma cell line used to fuse with the spleen cells was the NS1 cell line obtained from ATCC. The cells were grown up in 75 cm^2 flasks in RPMI 1640 with 15% fetal calf serum (GIBCO, RPMI W/FCS) in 7% CO_2 at 37°C. The cells were split 1:1 3 days in a row before the fusion to make sure they were actively dividing. Six flasks were pooled and the cells spun down at 160 x g for 5 minutes, then resuspended in 12 ml RPMI. Three ml aliquots were overlaid on 3 ml of Histopaque 1077 (Sigma) in a 15 ml centrifuge tube. These were spun in the centrifuge at 200 x g for 5 minutes. This procedure separates the live cells from the dead cells by leaving the live cells at the RPMI/Histopaque interface while the denser dead cells are forced into the Histopaque layer. Using a paster pipette,

the interface was removed and resuspended in 10 ml RPMI. The cells were spun down to wash off any excess Histopaque and resuspended in 20 ml RPMI. They were counted using trypan blue and a haemocytometer and gave a count of 6.5×10^6/ml.

In order to soften the blow for the newly fused cells trying to grow in a microtitre plate, a layer of what are termed "feeder cells" are mixed in with the fused cells. For our experiments, thymocytes were used as the feeder cells. Essentially, the thymus from one 4-6 week-old Balb/C female mouse was enough to supply the feeder cells for two 96 well microtitre plates. The thymus was broken up and washed in the same way as the spleen. In the case of the J fusion, 12 mice gave a feeder cell count of 1.8×10^8/ml with a total of 20 ml in RMPI W/FCS.

Once the three separate cell preparations were ready, the fusion process could be started. The cells were combined in a ratio of five spleen cells to one myeloma cell to a maximum of 10^8 cells in a 50 ml round bottom glass centrifuge tube. The cells were pelleted at 160 x g for 5 minutes and the supernatant discarded. For the J fusion, there were enough cells to have seven such tubes. To each tube, 1 ml of 40% polyethylene glycol (PEG, Hybriprep, GIBCO) in RPMI was added drop wise and the cells gently resuspended. The tubes were centrifuged at 250 x g for 10 minutes and the supernatant removed. To this, 10 ml of RMPI W/FCS was added to wash the cells and centrifuged at 160 x g for 5 minutes. The supernatant was discarded and the pellet gently resusupended in 39 ml RPMI W/FCS + HAT (Appendix A). Each tube had .55 ml of the thymocyte suspension added as the feeder layer giving about 2.5×10^6 cells/ml. This total cell suspension was plated at 200 ul per well of a 96 flat blottomed microtitre plate. For the J fusion, 14 plates were produced.

The plates were fed at four day intervals with RPMI W/FCS + HAT. Once the majority of wells were 50% confluent with hybridoma cells, the supernatants were screened for antibody. If a well was chosen to be minicloned, the cells were removed, counted and plated at 500-700 cells per 96 well microtitre plate using RPMI W/FCS and HT along with 2.5 x 10^6 thymocytes/ml. This set of plates would again be fed every four days until the clones were 50% confluent. The cell supernatants were tested as before and this time, the cells were cloned at 30-50 cells per 96 well microtitre plate. This is the final cloning step which should result in a production of a monoclonal antibody.

It is relatively easy to produce fused cells; the difficult part is deciding which hybridomas to clone to purity. Two methods were used to screen the wells for clones which should be expanded. One was a standard ELISA procedure while the other consisted of adding cell supernatants to nitrocellulose membrane strips with resolved chlamydial proteins.

The ELISA procedure used whole EBs as antigen for detecting antibodies in the culture supernatants. Approximately .25-.50 ug of EB protein in 50 ul of .01 M carbonate buffer pH 9.4 was added to each well of a 96 well ELISA plate. The plate was incubated overnight at 37°C in a moist atmosphere. The suspension was discarded and the wells blocked with 75 ul/well of 3% BSA in PBS. Following one hour incubation at 37°C, the BSA was discarded and 50 ul of the culture supernatants were added and incubated one hour at 37°C. This was then discarded and the wells washed three times (150 ul/well) with 1% BSA in PBS and then 50 ul/well of a 1:3000 dilution of rabbit anti-mouse IgG (HRP-labelled, DAKO) was added and allowed to incubate at 37°C for one hour. This was then discarded and the wells washed three times with plain PBS. The wells were developed by adding 50 ul/well of .8 mg/ml solution of 2,2'-azinodi-(3-ethylbenzthiazoline

sulfonic acid) (ABTS, Sigma) in .1 M citrate buffer pH 4.2 with 1 ul/ml 3% H_2O_2. Positive wells would turn dark green while negative wells were clear or faint green.

The ELISA procedure allows identification of antibodies which react with the homologous epitope as it is found on the EB. As whole EBs were used, any antibody would presumably react only with surface exposed epitopes. A second method of identifying reactive clones was used to complement the standard ELISA procedure. With it, we were able to isolate clones producing MAbs against a number of epitopes which would have been missed due to the numerous epitopes of the LPS and MOMP on the outer surface of the EB.

The procedure involves transfer of chlamydial proteins to nitrocellulose membrane, then reaction of the NCM with culture supernatants. EBs of the serovar with which the mice were immunized were solubilized and run on 10% PAG. The gels were electroblotted as described previously. The NCM was blocked with 4% BSA, then cut into 6 mm strips. Each strip was numbered and immersed in 5 ml TRIS/saline/Tween 20 buffer with 50 ul of culture supernatant from a hybridoma well with at least 50% confluency of the cells. The strips were incubated 1-2 hours at 37°C and then processed as described previously for NCM reacted with mouse antibody. Immune mouse sera was used as a positive control as well as a marker to establish what protein a given culture supernatant was reacting with, if it was positive.

9. Purification of Monoclonal Antibodies on Protein A Sepharose Columns

Protein A sepharose columns were used to purify and concentrate MAb found in culture supernatants and ascites fluid. Both of these sources of MAb are heavily contaminated with miscellaneous proteins found in the fetal

calf serum of the growth media or the serum components which mix with the ascites fluid obtained from the mouse peritoneal cavity.

To purify MAb from culture supernatants, the hybridoma cells were grown in 100 ml of media until the majority of the cells were dead, thus giving maximum MAb production. The cell debris was spun down and dialyzed overnight against .05 M TRIS/.15 M NaCl, pH 8.0 before being applied to the column.

To induce ascites formation, six week old Balb/C female mice were initially primed with either .5 ml pristane injected intraperitoneally and rested one week or injected with .5 ml Freunds incomplete adjuvant intraperitoneally and rested one or two days. After resting, the mice were then injected intraperitoneally with .5 ml RPMI containing 1-5 x 10^6 hybridoma cells. Within 8-14 days, the peritoneal cavity of the mice would swell with fluid due to the tumor growth. At this time, an 18 guage needle was inserted and the fluid drained into a sterile test tube. Usually, 10-15 ml would be obtained per mouse if they were drained twice in three or four days. The fluid is rich in antibody but also contains cells and other debris which is removed by a high speed spin at 10,000 x g for 20 minutes. The ascites fluid was diluted 1:10 with .05 M TRIS/.15 M NaCl pH 8.0 before running onto the protein A column with no more than 2 ml ascites fluid added to the column at any one time.

The protein A sepharose was obtained from Pharmacia in dry form of which 5 g was allowed to equilibrate in starting buffer (.05 M TRIS/.15 M NaCl pH 8.0). The slurry was then poured into 1.5 cm x 5.0 cm Econo-Column (Bio-Rad) and buffer allowed to run through to establish a steady flow rate. In the case of the dialyzed culture supernatant up to 200 ml would be run over the column while 20 ml of the diluted ascites fluid would be run at any one time. Starting buffer was used to wash off any unbound pro-

tein until the optical density (O.D.) at 280 nm of the wash was zero. To remove the bound antibody, a buffer of .1 M acetic acid/.15 M NaCl was run over the column. Two ml fractions were collected into 2 ml of .5 M phosphate buffer pH 8.0 to immediately neutralize the acid. The fractions were monitored for protein content at 280 nm and the antibody containing fractions were pooled. The column was washed with starting buffer and stored in the same buffer with .02% Na azide at 4°C.

10. Isotyping of Monoclonal Antibodies

This procedure was developed in our laboratory giving fast and clean cut results. Rabbit antisera against the various IgG isotypes (1, 2a, 2b, 3) was purchased from Miles Laboratories. The different sera were diluted 1:5 in .01 M TRIS/.15 M NaCl, pH 8.2 and 50 ul was allowed to bind for 30 minutes at R.T. to a NCM in a Bio-Dot (Bio-Rad) apparatus. Each piece of NCM would have the four anti-isotypes represented. The NCM was blocked with 4% BSA in TRIS/NaCl and 1 ml of a 1/50 dilution of MAb in TRIS/NaCl/.2% Tween 20/.1% BSA was added for 1 hour at 37°C. The second antibody of rabbit anti-mouse Ig (HRP) at 1:3000 was added for 1 hour at 37°C and then the NCM was developed. One well was left blank as a negative control to look for non-specific binding of the MAb. A positive control consisted of isotyping a MAb which had been previously isotyped by us and other laboratories by other methods.

11. Microimmunofluorescence (MIF)

This procedure was used to test human and mouse serum and MAb preparations for ability to react with C.trachomatis serovars, MoPn and CpMn. The actual testing was done by either R. Peeling or J. McDowell after a method by Wang et al (1982).

Essentially, chlamydial strains were grown in HeLa cells and harvested to the point where they were pelleted through a 35% renograffin gradient. The pellet was resuspended and formaldehyde was added to a final concentration of .2%. Aliquots of this material were mixed with a 5% yolk sac preparation and this suspension of EBs was then used to prepare slides for the MIF test.

Using Hunt's Finest Pen nib #104 secured to a pair of forceps, the yolk sac preparation was dotted onto a grease free microscopic slide in a predetermined grid pattern resulting in all C.trachomatis serovars being represented and MoPn and CpMn being added when desired. The slides were air dried and then fixed in acetone. The slides were now ready to have the antibody samples applied.

The grid of dots can be covered by a single drop of serum or MAb which has previously been diluted. Once all the various grids are covered with different samples, the slide is incubated at 37°C for 1 hour in a moist chamber. The slide is washed in PBS and distilled water, then fluorescein labelled anti-human Ig or anti-mouse Ig is added as a drop and the slides again incubated for 1 hour at 37°C. Following a wash step in PBS and distilled water and air drying, the slides were mounted with FA mounting fluid and viewed under 400x magnification using a Leitz UV microscope.

To titre immune sera, the highest dilution of sera to show fluorescence on any of the dots was considered that end point. The amount of fluorescence for each dot was also estimated on a 1+ to 4+ grading system. In the case of the MAb preparations, the titre was not so important as compared to what serovars the MAb reacted with, if there was any reaction at all.

12. Preparation of Affinity Columns

The idea of an affinity column is to fix a MAb to an insoluable matrix and have the MAb pick out its homologous protein from a mix of proteins passed over the matrix. The MAbs which were used were first purified on protein A sepharose columns and then reacted with CN.Br activated sepharose 4B (Pharmacia) essentially following the manufacturers instructions. This procedure would permanently fix the MAb to the sepharose beads.

As an example, a description of the process used to couple the MAb 2H9.G5, which reacts with a 29 Kd protein, will be related. The antibody was purified from acites fluid then dialyzed against .1 M carbonate/.5 M NaCl pH 8.3. The protein content was 350 ug/ml with a total of 8 ml. It is suggested by the manufacturer that between 1 and 10 mg of protein be bound to each gram dry weight of CN.Br-activated sepharose 4B. In this case, 2.80 mg of protein was bound to .5 g of sepharose.

The CN.Br-activated sepharose 4B (.5 g) was washed with 300 ml of 1 mM HCl on a scintered glass filter over a period of 15 minutes. The sepharose was then added to the 8 ml of MAb in .1 M carbonate/.15 M NaCl pH 8.3 in a 15 ml polystyrene centrifuge tube and rotated end over end for two hours at R.T. The excess ligand was removed and the sepharose washed with 100 ml of the carbonate buffer on the scintered glass filter. To block any remaining active groups, the sepharose was mixed end over end in 20 ml of .1 M TRIS pH 8.0 for 2 hours at R.T. This buffer was then removed by filtering on a scintered glass filter. The sepharose was washed with three alternating washes of low and high pH buffers. 30 ml of .1 M acetate/.5 M NaCl pH 4.0 and 30 ml .1 M TRIS/.5 M NaCl pH 8.0 were used each time and then the sepharose, with attached MAb, was stored in .1 M TRIS/.15 M NaCl pH 8.0 with .02% Na azide.

13. Fractionation of Chlamydial Proteins with Detergents

In order to purify a specific protein using affinity columns or carry out protease treatment of the MOMP, the chlamydial EBs had to be disrupted and the proteins solubilized. Caldwell et al (1981) described a method of producing almost pure MOMP using a two detergent system of breaking up the chlamydial EB. The method described below is an adaptation of Caldwell's work.

Purified EBs with a protein content of about 2.5 mg were suspended in 15 ml of .01 M phosphate/.15 M NaCl/1.5 mM EDTA pH 8.0 (EDTA buffer) and pelleted at 30,000 x g for 30 minutes. The pellet was resuspended using a 20 guage spinal tap needle in 5 ml of the above buffer and sarkosyl added to a final concentration of 2%. This was incubated for 2 hours at 37°C with end over end mixing. This solution was then spun in a type 42.1 rotor (Beckman) at 100,000 x g (35,000 rpm) in a Beckman ultracentrifuge for 60 minutes at 20°C. The supernatant was saved (Sarkosyl soluble, SARC. SOL.) and the pellet was washed once in 2% sarkosyl in EDTA buffer. The pellet was then resuspended in 2.5 ml EDTA buffer and SDS added to 2%. This suspension was incubated at 60°C for 2 hours with rotation and spun in an ultracentrifuge as described above. The supernatant was saved (SDS soluable, SDS SOL.) and the small amount of insoluable material (SDS insoluable, SDS INSOL.) left resuspended in 1 ml of EDTA buffer. These three different fractions of the chlamydial EB were then used for purification of specific proteins.

14. Treatment of Whole EBs With Dithiothreitol (DTT)

This procedure was used to try and extract any proteins which might be held to the chlamydial outer membrane by disulfide bonds. We initially used 200 ul (approximately 50 ug protein) of EBs (serovars L2 and D) and

added 800 ul of .05 M TRIS/.15 M NaCl/20 mM DTT, pH 7.8. This was incubated at 37°C for 1 hour. The suspension was spun in a microfuge for 15 minutes and the supernatant transferred to another tube and spun again. Aliquots (20 ul) of each supernatant were run on PAG and silver stained, or they were brought to 1 ml with TRIS/SALINE buffer and injected into 1 Balb/C female mouse, each using the same injection schedule as for hybridoma production.

To try and increase the amount of protein in the supernatant fraction, 800 ul of EBs (serovar L2 only; approximately 200 ug protein) were spun down and resuspended in 800 ul TRIS/SALINE/DTT using a 27 guage needle. The suspension was incubated for 30 minutes at 37°C and then spun in the microfuge. The supernatant was transferred to another tube and spun again. It was then brought to a 3 ml volume (total 40 ug protein) with TRIS/SALINE and injected into two Balb/C female mice as before. The sera in both sets of mice were used in immunoblots against whole EBs (serovars L2 and D).

15. Isolation of Chlamydial Proteins Using Affinity Columns

The three protein fractions resulting from the detergent solubilization of chlamydial EBs were used to isolate specific chlamydial proteins by reaction with affinity columns with MAb fixed to them.

When a protein found in the SARC. SOL. fraction was to be purified, the sarkosyl was first removed by dialysis against two changes of .05 M TRIS/.15 M NaCl/.05% NP.40 (Sigma) pH 7.5 (NP.40 buffer) at 4°C. The SARC. SOL. fraction after dialysis (about 10 ml) was added to the MAb-sepharose in a 50 ml centrifuge tube and rotated for 24 hours at 4°C. The sepharose was then poured into a 1.5 cm x 5.0 cm Econo-Column (BIO-RAD) and washed with 200 ml of the ice cold NP.40 buffer. The protein was eluted

with .5 M acetic acid/.15 M NaCl collecting .5 ml fractions into .5 ml .5 M phosphate buffer pH 8.0.

Essentially, the same process was used for the SDS SOL. fraction except the total volume would be brought to 10 ml with NP.40 buffer.

The SDS INSOL. fraction was handled differently in that the protein was not initially in a soluble form. It was felt that there was a high degree of disulfide bonding between the proteins found in this fraction which would have to be broken before the protein would solubilize. The SDS INSOL. fraction (1 ml) which has some residual SDS had 2 mercaptoethanol added to a concentration of 1 mM. The suspension was boiled for 10 minutes and then 9 ml of NP.40 buffer was added with the total of 10 ml mixed with the MAb-sepharose at 4°C for 24 hours.

In general, 20 x .5 ml fractions were collected when the protein was diluted from the affinity column. The column volume was usually less than 3 ml so collection of 10 ml caught any protein released from the column. To determine where the protein was 20 ul of 100% trichloroacetic acid (TCA) was added to 180 ul of each fraction, mixed and put on ice for 10 minutes. This was spun in a microfuge for 10 minutes and the supernatant discarded. 100 ul of acetone was added, vortexed and iced for 10 minutes. This was again spun for 10 minutes, the acetone removed and the tube allowed to air dry. 25 ul of solubilization buffer was added and boiled for 5 minutes. A 20 ul aliquot of this was run on a 12% PAG in a mini-gel system (BIO-RAD) and the gels silver stained. The fractions (usually numbers 5-12) which contained protein were pooled, dialyzed against .05 M TRIS/.15 M saline and prepared for injection into mice to produce monospecific polyclonal sera.

16. Protease Digestion of Chlamydial EBs

Proteases are enzymes which recognize a specific amino acid or group of amino acids and cleave the protein at that site. When a protein is in its natural conformation in the intact organism, none or only a few of the protease sites would be available for cleavage as compared to the purified form of the protein. Comparison of the two peptide maps generated using intact EBs and semi-purified soluble MOMP should help in developing how the MOMP fits into the outer membrane. Protease digestion could also be used as a tool to decide whether or not a protein is surface exposed and, if the protein is cleaved, then it must have a protease sensitive site which is surface exposed.

Whole chlamydial EBs were diluted in .05 M TRIS/.15 M NaCl pH 7.8 and had varying amounts of the protease staphylococcal V8 protease (Miles), trypsin (B.M.) and chymotrypsin (B.M.) added to them. The reactions were carried out in microfuge tubes at 37°C. Aliquots were removed at various time periods and phenylmethylsulfonyl fluoride (PMSF) added to final concentration of 2 mM and, in some cases, an equal volume of solublilization buffer. After boiling the sample for 5 minutes, it was run on PAG and the gel either silver stained or electroblotted. In other cases after the PMSF was added, the protease treated EBs would be used to infect HeLa cell monolayers to determine whether or not the EBs were still viable.

17. Isolation of MOMP Peptides

We combined the protease digestion of whole EBs followed by their solubilization with 2% sarcosyl to isolate MOMP peptides. The EBs were treated with trypsin for 15 minutes and then PMSF was added and the suspension spun for 30 minutes at 15,000 rpm. The pellet was resuspended and handled as before for isolation of the chlamydial outer membrane. The

trypsinized outer membrane was solubilized and run on PAG for silver staining. As well, the separated peptides were transferred from the PAG to activated glass fibre to allow amino acid sequencing after a method described by Aebersold et al (1986). The peptides were also separated using HPLC essentially following the method of Judd and Caldwell (1985).

18. Infection of HeLa Cell Monolayers on Coverslips

In order to count the number of inclusion forming units (IFU's) there are in a sample of EBs, the preparation must first be diluted and then aliquots of each dilution inoculated onto a set number of cells and the inclusions counted 48-72 hours later. The system used in our laboratory is one originally developed by Furness et al (1960) and performed by either R. Peeling or J. McDowell.

HeLa cell monolayers were first grown up on glass coverslips. A flask of cells was trypsinized and an aliquot of the cells counted. The cells were diluted in MEM such that 1 ml contained 4.5×10^5 cells. Sterile glass dram vials with a 13 mm coverslip in the bottom had 1 ml of the cell suspension add to them and were then incubated at 37°C overnight to allow development of a cell monolayer. The next day, the MEM was removed by suction and the cell monolayer washed once with .5 ml HBSS. The various dilution of EB preparations were added in .1 ml volumes to each vial and the vials sat at room temperature for 1 hour to allow uptake of the EB. The inoculum was then suctioned off and the monolayer washed once with .5 ml HBSS and then 1 ml of MEM/cycloheximide was added. The vials were incubated at 37°C for 48 hours.

To visualize the inclusions, the medium was removed from the coverslips and they were washed once with PBS. Then .5 ml of 20% methanol was added to fix the cells. The coverslips were removed from the vial, fixed

in an acetone bath, washed three times in PBS and then 7 ul of Syva stain (Syva Corporation*) was added to the cell side and the coverslip was inverted onto a microscope slide. The slides were incubated for 1 hour at 37°C and then thirty fields counted using a UV microscope.

19. Neutralization Assay

Essentially, this procedure was the same as the infectivity assay, except the EB preparations were first reacted with immune sera or purified MAb before being inoculated onto the cell monolayer.

Predetermined numbers of EBs were mixed with the immune sera or MAb and incubated at 37°C for 30 min. Residual infectivity of the reaction mixture was assayed on 24-hour HeLa 229 monolayers. A positive control would consist of an EB preparation with no immune sera or MAb added, while the negative control would consist of pre-immune sera or purified Ig from pre-immune sera.

20. Iodination of the MOMP

The source of the MOMP was the SDS SOL fraction from serovar L2. It had a protein concentration of approximately 200 ug/ml. Two iodobeads (Pierce Chemical Co.) were washed in PBS for 5 minutes at room temperature. To this was added 100 ul SDS SOL fraction (approximately 20 ug protein) in 400 ul PBS. The reaction was started by the further addition of approximately 1 mCi ^{125}I and allowed to continue for 15 minutes. The reaction was stopped by removal of the iodobeads. Free ^{125}I was separated from the MOMP by running over a Sephadex G-25 (Pharmacia) column in PBS/.1%

* Syva stain is a fluroescent labelled monoclonal antibody which reacts with the MOMP of all C.trachomatis serovars.

SDS. The column was 5 ml volume in a 5 ml syringe. Fractions of .5 ml were collected. Aliquots of each fraction were run on PAG, immunoblotted and autoradiographed.

To determine how much of the total count was bound to protein, aliquots of each fraction were precipitated with TCA as follows:

	.5% BSA	Sample	100% TCA
TCA +	895 ul	5 ul	100 ul
TCA -	995 ul	5 ul	-

Each sample was iced for 15 minutes, then spun in a microfuge for 5 minutes. 100 ul of each supernatant was counted.

$$\% \text{ TCA ppt} = \frac{(\text{CPM TCA}^- - \text{cpm TCA}^+)}{\text{cpm TCA}^-} \times 100$$

Fractions 5, 6 and 7 had the highest values of 93.9%, 92.6% and 90.9% respectively and were pooled. This labelled MOMP was then used for peptide mapping.

21. Iodination of Whole EBs

This procedure was used to iodinate surface exposed proteins. 250 ul of serovar L2 EBs (approximately 100 ug protein) in SPG were spun down in a microfuge. The pellet was resuspended using a 2 guage needle in 300 ul PBS. To this were added two iodobeads and 1 mCi ^{125}I and the reaction was allowed to go for 15 minutes. The suspension was then transferred to a microfuge tube and spun for 15 minutes. The supernatant was discarded and the pellet resuspended in 250 ul of SPG. Aliquots were run on PAG, immunoblotted and autoradiographed.

RESULTS

1. Comparison of the Protein Profiles of Chlamydial Serovars using SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The purified EBs of the 12 serovars, the MoPn and the C.psittaci Mn strains were solubilized by boiling with SDS and 2-mercaptoethanol and then the individual proteins were separated on either 10% or 12% PAG. The 10% gels tended to allow better resolution of the protein with molecular weights (MW) >40 Kd but the dye front would run around 20 Kd so proteins in this area were not visualized. A 12% PAG, while tending to crowd higher MW proteins together would allow clearer resolution of proteins from 40 Kd down to around 10 Kd.

Once the protein components had been separated, the gels were stained in order to see the protein bands. In earlier reports, both Coomassie brilliant blue (CBB) and autoradiography (with ^{14}C-amino acids) were used to visualize chlamydial proteins. The first technique tends to be quite insensitive unless large amounts of protein are available while the second method is relatively expensive and lacks the resolution of specific protein bands seen with CBB staining. Our initial attempts at staining chlamydial proteins with CBB revealed only a few bands with MW ranging from 40-100 Kd in much the same picture as the gels of Caldwell and Judd (1982) and Newhall et al (1982). For this reason, we settled on the use of a silver staining method devised by Morrissey (1981) allowing us to use about 2 ug of total EB protein for each lane on the PAG. Depending upon the length of the development process, very minor proteins could be seen.

On a given PAG run, each lane was loaded with approximately 2-5 ug of total protein following solubilization and then separated into the individual protein bands numbering anywhere from 50 to 100. An example of a 12% PAG used to separate the proteins of a number of chlamydial strains then

stained with silver is seen in Figure 1. This gel shows the protein profiles of 12 C.trachomatis serovars, the C.trachomatis MoPn strain and the C.psittaci Mn strain.

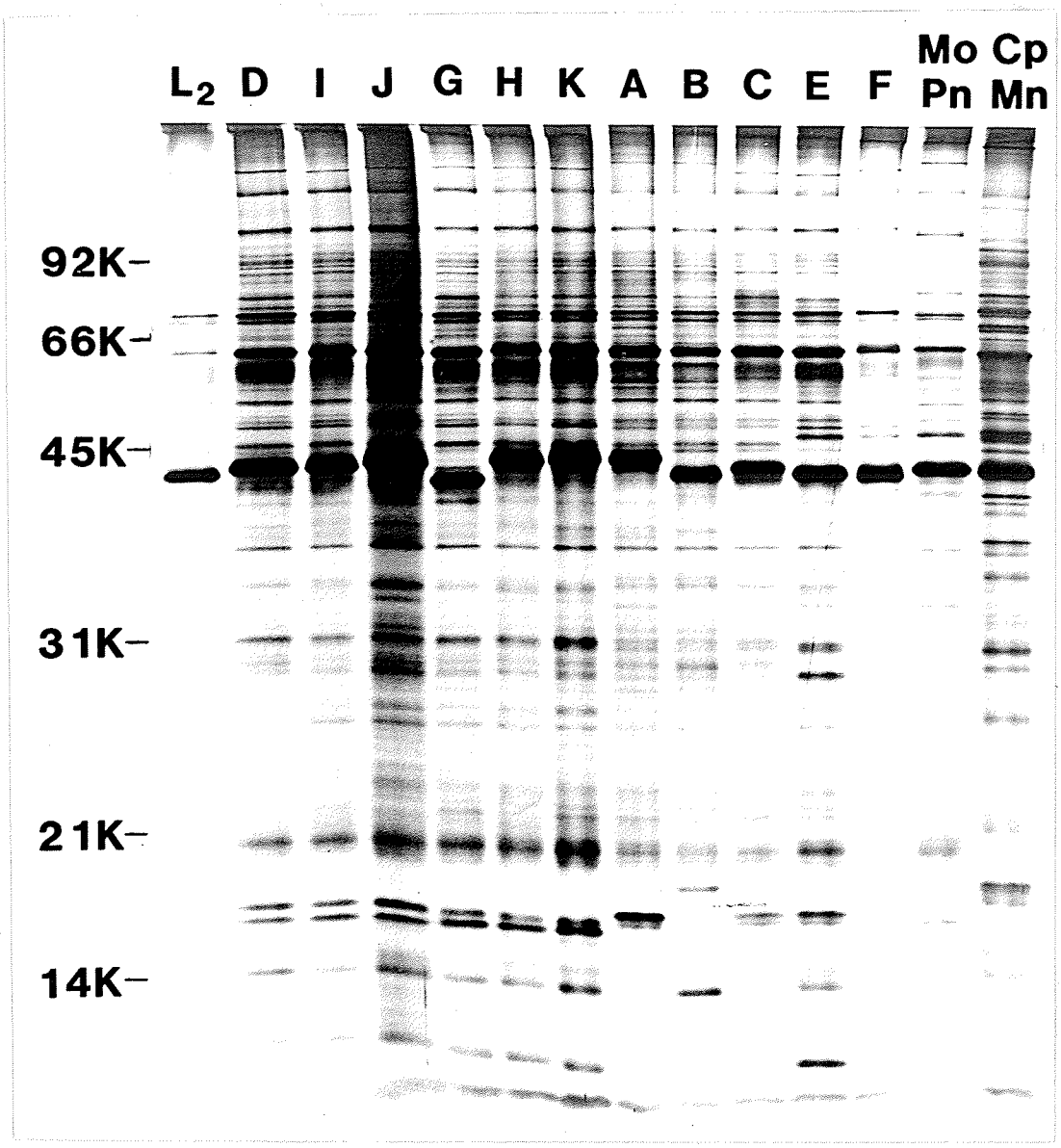
The most striking feature of the protein pattern is the dominant band at 40 Kd. It is found in all of the chlamydial strains but is seen to have a variable molecular weight depending upon the strain. This protein has been previously named the major outer membrane protein (MOMP) by Caldwell et al (1981). In the lane containing serovar L2 protein (which has been under loaded), the MOMP is essentially the only protein seen except for a few higher MW proteins.

The next most prominently stained proteins are 57 Kd and 60 Kd. These appear common to all C.trachomatis serovars but only the 60 Kd protein is perhaps common also to the MoPn and CpMn strains. These two proteins make up what Newhall et al (1982) has termed the 60 K doublet. His molecular weight estimates for these proteins are 60 Kd and 62 Kd.

As seen in the serovar L_2 lane, there is a protein band at approximately 75 Kd which has equal staining intensity as the band at 60 Kd. The 75 Kd band is common to all C.trachomatis serovars with no variation in molecular weight as is seen with the MOMP. Protein at a similar molecular weight are seen in the MoPn and CpMn strains.

While many minor protein components appear common to all the serovars, some variability does exist. At approximately 17 Kd, the serovars L2, D, I, J, G, H and K have a similar looking doublet. Serovars A, C, E and F have a different doublet of slightly higher MW with serovar B having no comparable doublet but only a single band in that area which is again of slightly higher MW. It is hard to say whether such bands exist in MoPn and CpMn. A single band at 17 Kd is seen with MoPn while CpMn has a doublet with the major band closer to the size of the serovar B protein.

FIGURE 1. Polyacrylamide gel (12.5%) of 12 serovars of C.trachomatis, the mouse pneumonitis (MoPn) strain and the C.psittaci (CpMn) strain stained with silver. The serovar designations are listed across the top of each lane with the molecular weight estimates given on the left.



Differences also exist in the apparent amounts of individual protein bands. A comparison of lanes with serovars C and E which have equal amounts of protein loaded shows serovar E with increased amounts of material at 10 Kd, 30 Kd and 45 Kd. Whether these differences are real or due to minor difference in growth conditions cannot be determined.

In general, the 12 serovars show both major and minor protein components, some of which appear common to all, others being specific to single or a subset of serovars. Simply looking at the silver stained gels does not help in determining the function or importance of a given protein in the infectious process of the whole EB. To try and pin-point significant proteins, we turned to the analysis of the immune response to chlamydial protein during natural human chlamydial infection.

2. Immunoblotting of C.trachomatis Serovars with Sera from Individuals Naturally Infected with Chlamydia

As can be seen from the silver stained gels, the EBs of the various serovars are made up of a large number of different proteins. The problem is to decide which are significant in disease and should be studied in detail. Significant antigens were selected as those which were recognized during natural human infections.

We started by screening laboratory personnel with known titres to chlamydia as determined by MIF as well as those with no titres to act as controls. A second group included men with Reiter's syndrome which is thought to be induced by a chlamydial infection. The third group consisted of infertile and fertile women who had antibody titres to chlamydia. The immunoblotting was undertaken as part of a larger study to determine risk factors for infertility due to infectious causes (Brunham et al, 1985).

Sera were tested at a dilution of 1:500. Previously, control sera (MIF negative) had been used at varying dilutions from 1:100 to 1:1000. At low dilutions, the MOMP tended to non-specifically bind antibody. This was completely eliminated by diluting the immune sera at 1:500. The sera were blotted using a limited number of serovars as there was no use in looking at a complete panel at this time. The majority of cases were analyzed using serovars L2, D or I as antigen, depending upon the specificity suggested by the MIF results. We developed the immunoblots with rabbit-anti-human IgG, IgA and IgM. Examples of the immunoblots from the various groups listed above will be used to illustrate the varied immune response to chlamydial proteins.

The immunoblot of an individual from our laboratory with a subclinical, laboratory acquired infection with serovar L2 (MIF titre >1:1000) is seen in Figure 2. There is an intense response to the MOMP of serovar L2 and to a lesser extent to that of serovar D. At least 40 other antigens are recognized with the majority of those being greater than 40 Kd. There is some variability in the protein profiles at approximately 70 Kd, 55 Kd, 45 Kd, 32 Kd and 28 Kd. These are subtle differences between the serovars not easily seen when looking at silver stained gels alone. This immunoblot shows that while MOMP is a major immunogen, many other chlamydial antigens are also immunogenic during a chlamydial infection. Because this response was so extensive, it really does not help in determining important proteins. It does show that while the response to MOMP from different serovars is varied, response to the other antigens is constant. This would suggest that the MOMP has more epitope variability between serovars while the other antigens have little or no epitope variability between serovars.

An example of a less extensive response is seen in Figure 3. This is an immunoblot of serovars D and I developed with sera from a patient with

FIGURE 2. Immunoblot with sera (1:500) from laboratory worker subclinically infected with serovar L2. Lane A: serovar L2; lane B: serovar D. The whole EBs were solubilized and separated on a 10% PAG.

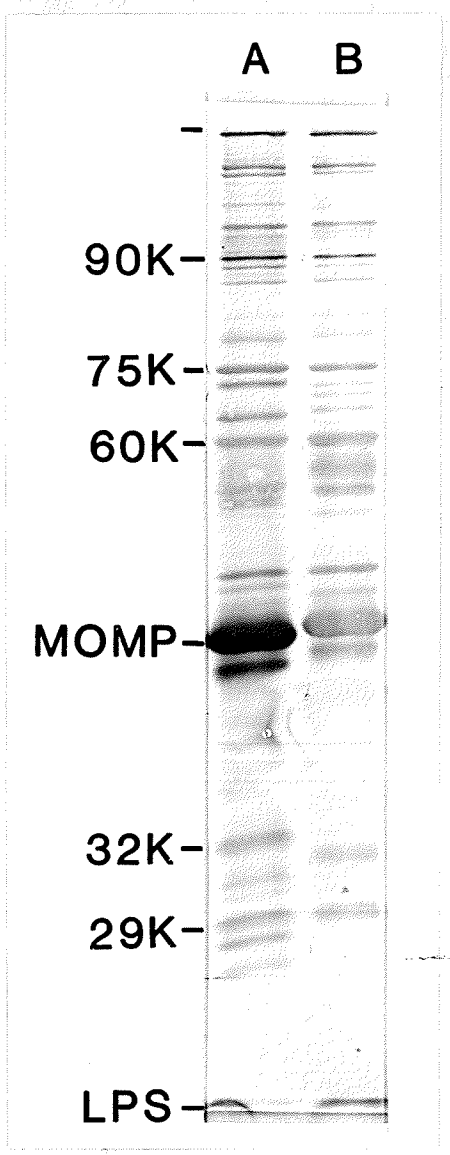
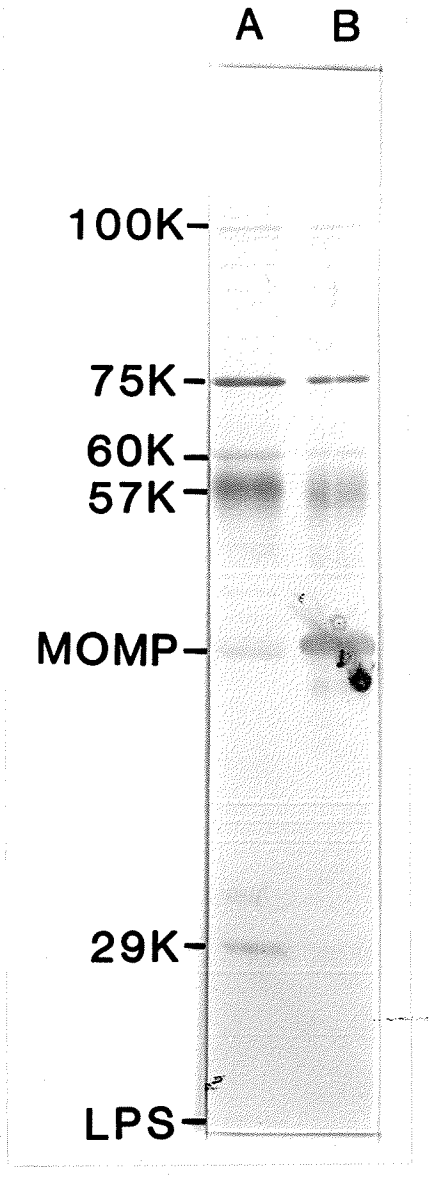


FIGURE 3. Immunoblot with sera (1:500) from patient with Reiter's syndrome infected with serovar I. Lane A: serovar D; lane B: serovar I. The whole EBs were solubilized and separated on a 10% PAG.



Reiter's syndrome. The homologous infecting serovar was felt to be serovar I as determined by MIF. The response to MOMP is almost specific for serovar I with minor binding to the MOMP of serovar D. Also seen with equal intensity is an antigen at 75 Kd and another at 57 Kd. The reaction of the 57 Kd antigen of serovar D is more intense than that of serovar I suggesting there may be more of that antigen present in serovar D than I. Only two antigens at 32 Kd and 29 Kd are seen below the MOMP while a few antigens at 60 Kd and approximately 100 Kd are also recognized. The only common response to both serovars is against the antigens at 57 Kd and 75 Kd.

The variability of individual responses to a chlamydial infection can be seen in Figure 4 (a, b, c, d). In Figure 4a, there is a strong response to MOMP as well as antigens of 57 Kd, 60 Kd, 75 Kd and approximatley 150 Kd. The next immunoblot (Figure 4b) shows a specific response to only MOMP and a 45 Kd antigen. Another example (Figure 4c) is essentially a MOMP specific response although other antigens are recognized but not in proportion to the MOMP. A complete turnaround from the previous immunoblots is seen in Figure 4d. Here, there is a generalized response to MOMP but the most extensive response is to antigens of 57 Kd and 60 Kd. There is also a limited response to two antigens around 30 Kd.

The immunoblotting allowed us to focus our attention on a number of specific antigens of C.trachomatis. From sheer prominence in silver stained gels, the MOMP stands out as an important protein and immunogen of chlamydia. However, the immunoblots demonstrated that antigens of 57 Kd, 60 Kd and 75 Kd may also be important. Their continual recognition during human infection may be due to surface exposure or the part they play in the pathogenesis of infection.

FIGURE 4. Immunoblot with sera (1:500) from four female patients with previous genital chlamydial infection showing the varied humoral immune response to different chlamydial antigens. Lane A: serovar L2; lane B: serovar D. The whole EBs were solubilized and separated on a 10% PAG.

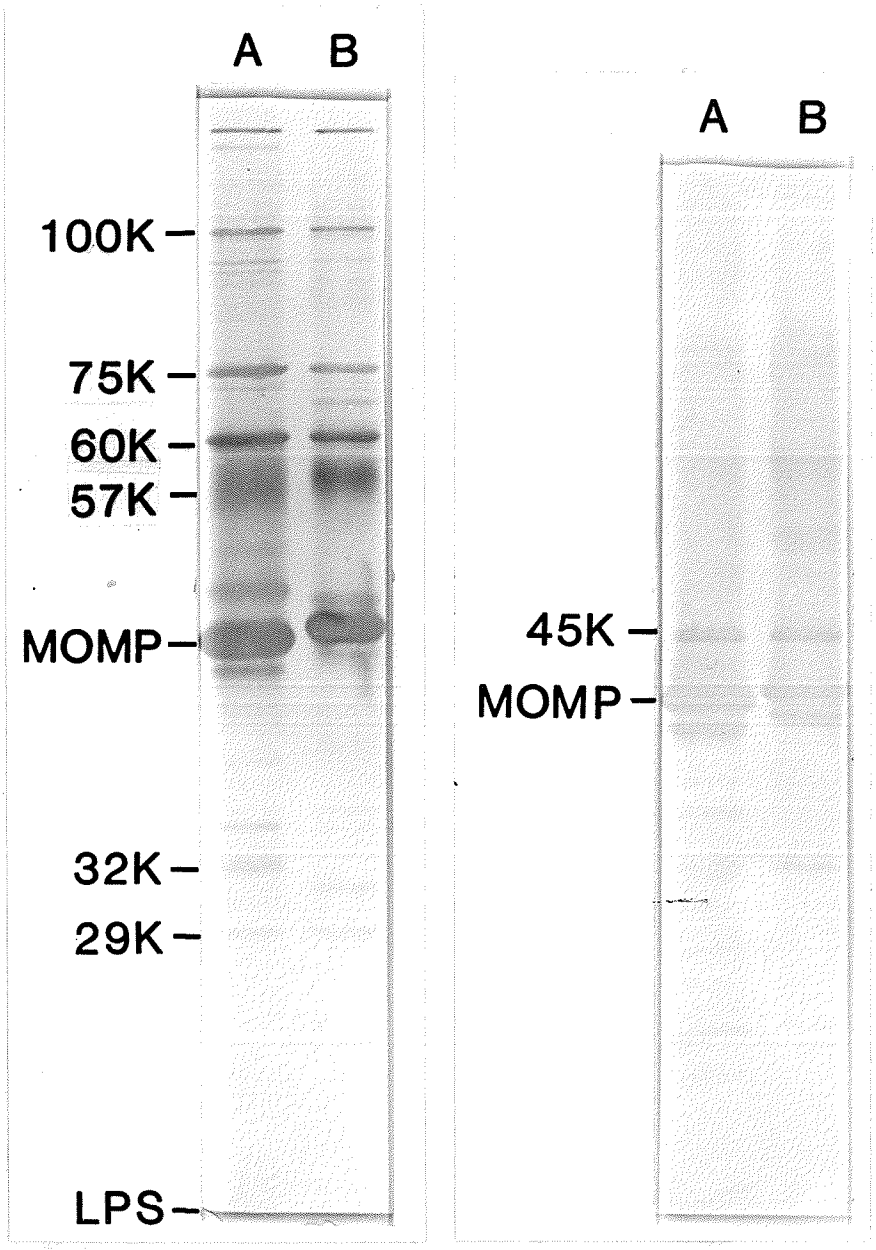
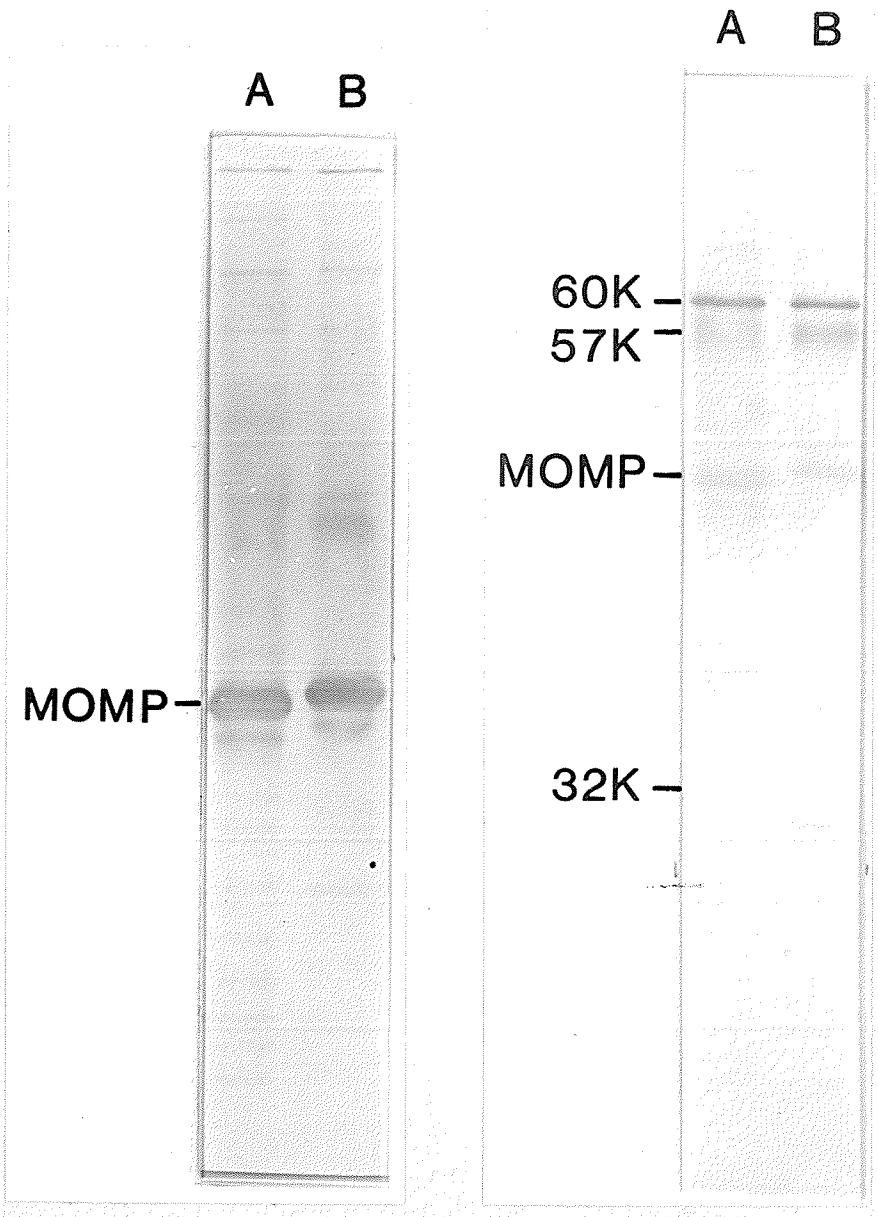


FIGURE 4. (continued)



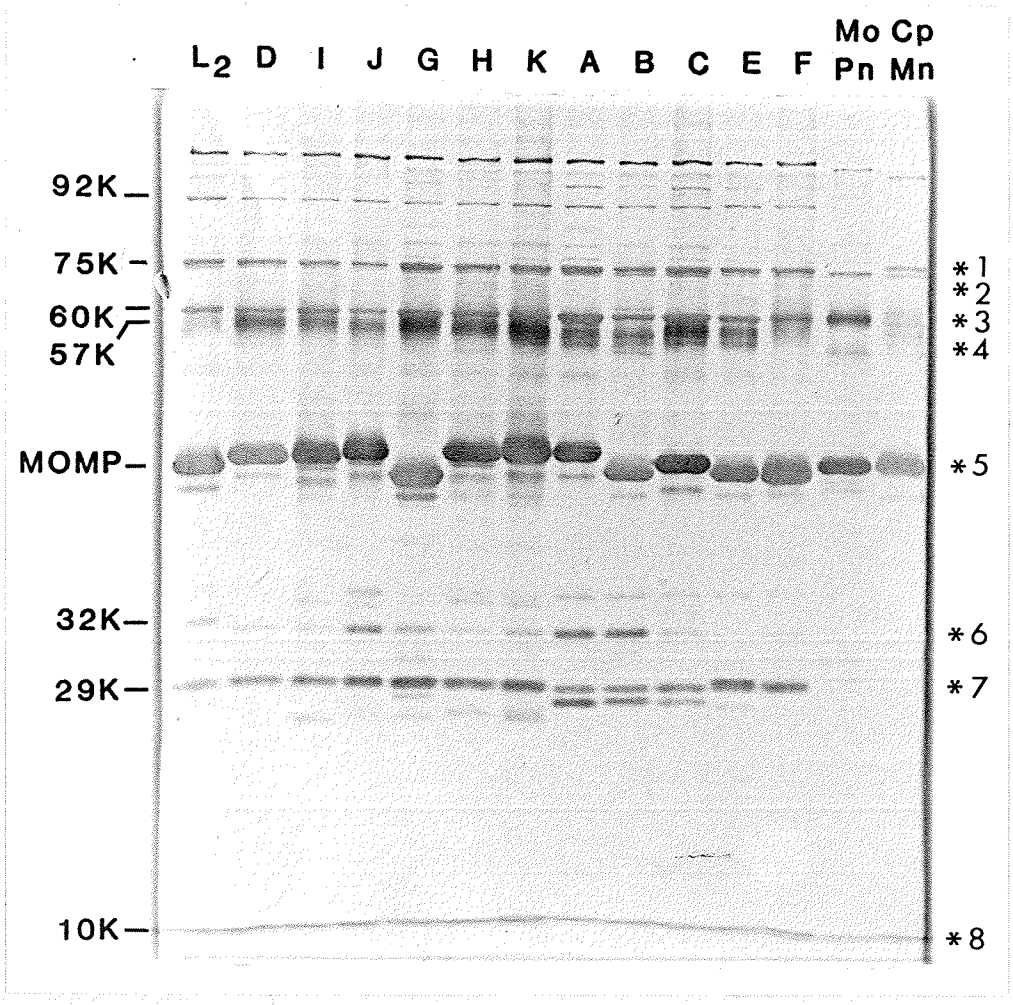
In 1982, MAbs had already been produced against the MOMP and LPS while the MOMP had been purified and some antigenic analysis carried out. For this reason, while not ignoring MOMP and the LPS, our interest was focused on trying to define the role of some of these other antigens. It must be remembered that while the MOMP is dominant in the outer membrane, making up around 60% of the protein, that still leaves approximately 40% to be made up by other proteins. We set out to produce the immune response of infected humans in mice so that we could raise MAb against some of these lesser antigens.

3. Analysis of the Serological Immune Response of Mice When Immunized With Whole EBs of C.trachomatis

Three sets of mice were immunized with whole EBs from C.trachomatis serovars L_2, J or G to be used in the generation of monoclonal antibodies. As an example of the sera obtained from these mice, an immunoblot developed with a 1:500 dilution of the serovar J immune sera is seen in Figure 5. A companion silver stain gel for this immunoblot is seen in Figure 1. The only difference is that the immunoblot was from a 10% PAG while the silver stained gel is a 12% PAG. A 10% PAG was used for the immunoblot as the majority of reactive epitopes were on high MW proteins. When a 12% PAG was used for the immunoblot, no reaction was seen with antigens less than 25 Kd (data not shown) other than the LPS.

The MOMP is seen as a variable band at about 40 Kd. The intensity of blotting is quite uniform among the serovars suggesting that the antisera have antibodies to species specific epitopes. This differs substantially from mice given only IV injections which tended to produce predominantly a serovar specific response (data not shown). This type of intravenous immunization was abandoned for this reason. Included in this immunoblot

FIGURE 5. Immunoblot (using J immune sera at 1:500) of 12 C.trachomatis serovars, the MoPn strain and the C.psittaci strain separated on a 10% PAG and transferred to NCM. Molecular weight estimates of the more prominant antigens are given on the left, with the starred numbers 1-8 on the right designating the antigens to which monoclonal antibodies were raised.



are the C.trachomatis MoPn and C.psittaci Mn strain. We were surprised to see a substantial amount of cross-reaction between these two strains and the C.trachomatis serovars. The MOMP was included among the cross-reactive antigens. This means that there are genus specific epitopes found on the MOMP common to the C.trachomatis serovars, the MoPn and the C.psittaci Mn strains. Whether there is only one common epitope or a number on MOMP cannot be determined from these data.

Another major immunogen seen on this immunoblot is an antigen at about 10 Kd. When a 12% PAG is seen, this antigen reacts at a lower MW, leading us to suspect it is the LPS. As can be seen, the reaction is genus specific giving an approximately equal amount of reactivity in each lane.

The immunoblot also shows a reaction with at least 20 other non-MOMP antigens. This suggests these antigens have at least one common epitope among the various serovars. Of note, there is a 100 Kd antigen which appears to have a common MW with the C.trachomatis serovars and a slightly reduced MW with the MoPn and C.psittaci Mn strains. An antigen at 92 Kd is common only to C.trachomatis serovars and is not seen in MoPn and CpMn. Another antigen which appears to be common to all chlamydial strains is at approximatley 75 Kd. This band is actually a doublet which on other PAG and immunoblots is seen as two bands of 75 Kd and 70 Kd. In the lane with the CpMn proteins, two bands are seen reacting with the serovar J sera. Only one band appears in the MoPn lane with a major band and a minor band seen in the other serovars.

The next major antigen is at 60 Kd. This protein has epitopes common to all the serovars and apparently the MoPn strain, but no comparable band is seen in CpMn. An antigen of slightly lower MW (approximately 57 Kd) is again common to all the serovars but is not seen at all in the MoPn and CpMn strains. There appear to be differences in the intensity of colour

development between the various C.trachomatis serovars. This suggests that some serovars may have more of this specific antigen than do other serovars or that there are subspecies specific epitopes on this antigen. As a comparison, there appears to be a greater amount of the 57 Kd antigen in serovars D, G, H, K, A and C, as compared to the other serovars. L_2 and F appear to have very little. Serovar J against which the sera was raised and which has the heaviest antigen load on the blot still has less of a reaction with the 57 Kd antigen than the other serovars mentioned. This would suggest that some serovars just have more of this antigen per EB than other serovars as opposed to differential distribution of subspecies epitopes.

This difference in colour development is again seen in an antigen at 32 Kd. It appears common to all serovars and is not seen in the MoPn and CpMn strains. Three serovars (J, A and B) are more intensely developed than the others, suggesting variable production of the antigen or the presence on this antigen of species and subspecies epitopes. There is a difference in MW with the antigen in L_2 being about 32 Kd while the other serovars have this antigen at a slightly lower MW.

A very interesting antigen is seen at 29 Kd. When run on a 12% PAG, this single band becomes a doublet. It is common to all serovars while comparable antigens in MoPn and CpMn are not seen. Remembering that the sera was raised against serovar J, the three antigens of serovars A, B and C seen just below the 29 Kd band become very interesting. These antigens must have epitopes which are found on a different MW antigen in the serovar J profile. An educated guess would be that the band at 29 Kd in serovars A, B and C is a single band, while in the other serovars, it is a doublet. The other half of the doublet for serovars A, B and C is the band at the lower MW. These three serovars are distinguished by being the traditional

trachoma serovars. Other than the obvious difference in the MW of their MOMP, this is the only other variation noted among their common antigens.

Although several antigens react strongly on the immunoblot, can anything be said about antigens which are weakly reactive or non-reactive? There are a number of antigens among the serovars which show an immunoblot reaction but no comment was made because the colour development was low and the response inconsistent. Examples of this are antigens reacting between 92 Kd and 100 Kd. The intensity is much less than the other antigens so it is more difficult to come to specific conclusions. Also of interest is the huge gap of any antigen reactivity between MW 29 Kd and the LPS. As can be seen in the silver stained gel (Figure 1), there are a substantial number of proteins present but they have apparently not stimulated the mouse immune system. The case could be made that if every antigen seen on the silver stained gel had homologous antibodies in the immune sera, then the EBs must have been degraded and the antigens processed individually. However, this is not what is seen. Only 20 or so of the approximately 100 macromolecules seen on the silver stained gel react in the immunoblot. This would suggest there is something unique about these antigens such that they induce an immune response. Interestingly, these are also the same antigens that are recognized during a natural human immune response to a chlamydial infection. It may be that these antigens are surface exposed while the non-immunogenic antigens are cytoplasmic proteins. They may also play key roles in the infectious process or just be present in larger amounts such that the immune system cannot miss them. As far as can be determined, the immunized mice never became infected or showed ill effects from the immunization schedule that was used. How they processed the whole EB's is not known.

Overall, this type of serological immune response duplicates that seen in humans with chlamydial infections and allowed us to isolate hybridoma cells producing monoclonal antibodies against these interesting antigens.

4. Production of Monoclonal Antibodies Directed Against C.trachomatis Antigens

The procedure to generate MAbs is used in laboratories around the world. The major differences in techniques tend to arise in the immunization schedules and the screening procedures. The immunization protocol depends upon what type of immune response is needed and what type of antigen is used. A variety of screening procedures allow the identification of specific MAbs which have unique characteristics.

In our laboratory, we undertook three separate cell fusion experiments. The immunization schedule was a variation of a previously published protocol by Stephens et al (1982). Initial attempts with shorter immunization schedules or that used only intravenous injections gave an immune response restricted to the MOMP. This was unsatisfactory for our purposes. The use of Freunds adjuvant and intraperitoneal and subcutaneous injections elicited a serological immune response similar to naturally infected humans.

The screening procedure initially used only a standard ELISA system in an indirect binding assay consisting of adding hybridoma supernatant to microtitre plate wells coated with whole EBs. This was used exclusively throughout the first fusion (serovar L_2) and tended to identify only anti-MOMP and anti-LPS MAbs. When the sera from these mice were immunoblotted, we could see that there were more antibodies present than those recognizing the MOMP and LPS. For this reason, we added an immunoblot screening procedure in the second and third fusions to complement the ELISA assay system.

FIGURE 6. Immunoblot using sera (1:500) from mice immunized with whole EBs from serovar L2. The whole EBs from the serovars listed across the top were solubilized and separated on 10% PAG. The MW of the most reactive antigens are listed on the left.

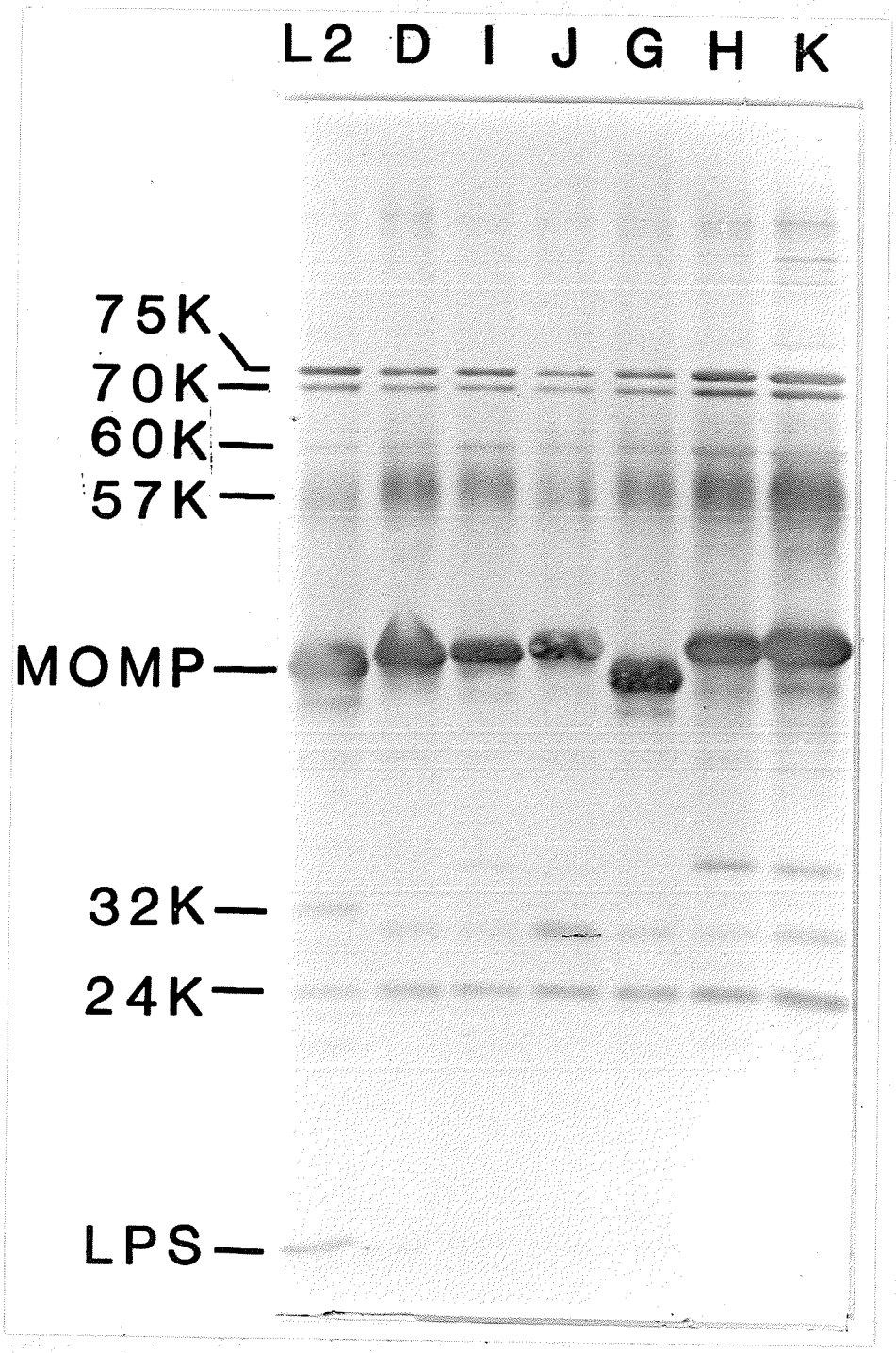
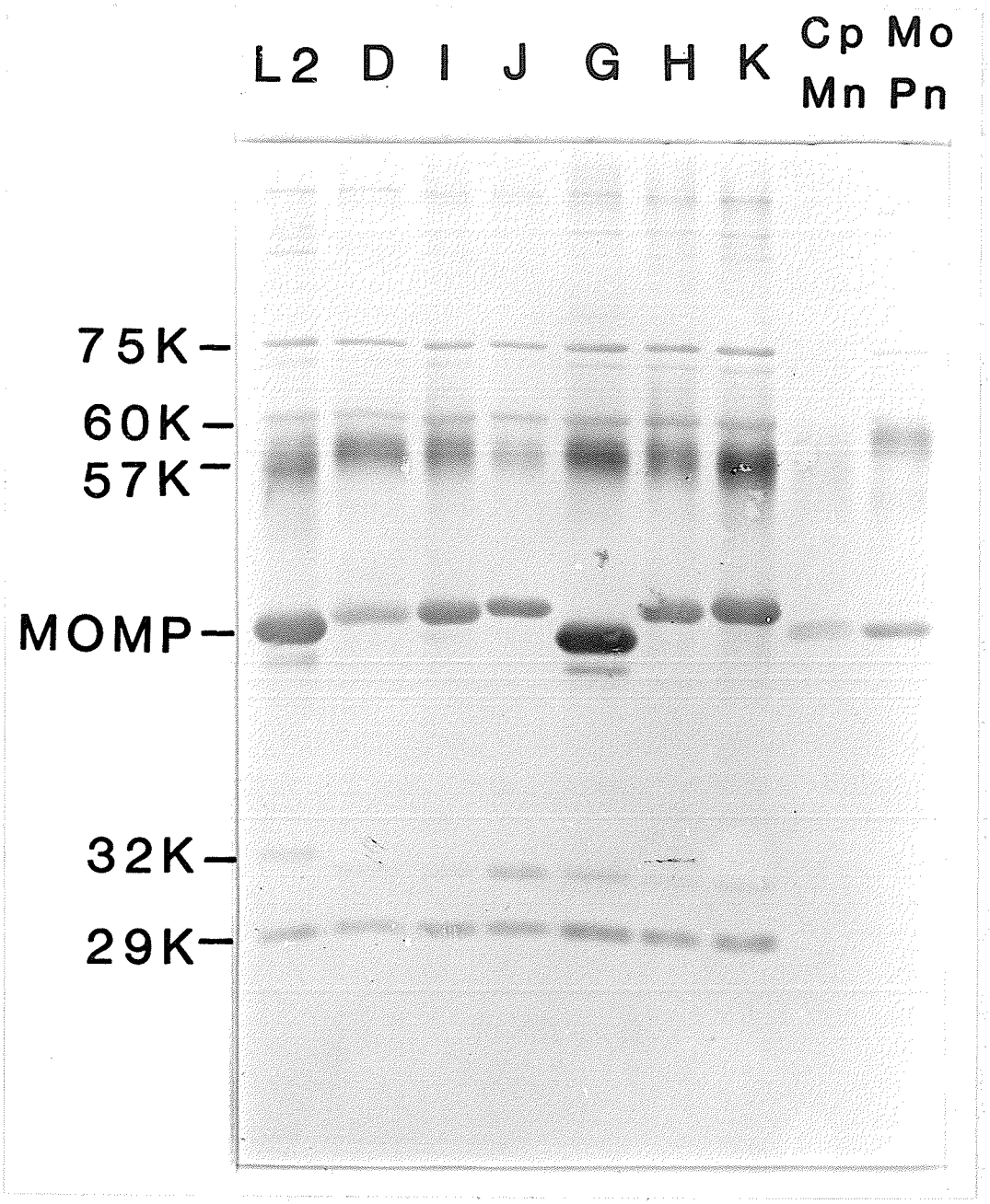


FIGURE 7. Immunoblot using sera (1:500) from mice immunized with whole EBs from serovar G. The whole EBs from the serovars and strains listed across the top were solubilized and separated on 10% PAG. The MW of the most reactive antigens are listed on the left.



The immunoblots using the sera from the three different fusions are seen in Figures 5, 6 and 7 for the serovar J, L_2 and G fusions, respectively. The sera react against essentially the same antigens showing that the immunization protocol was consistent. The most dominant response in all three is against the MOMP. There are variations, however, between the different sera. For example, the serovar L_2 serum has a better response against the 70 Kd antigen than the other two sera and the response to MOMP is much more uniform than that of the serovar G even though the G fusion serum has genus specific antibodies to MOMP. A summary of the results from the three fusions is given in Table 3.

Table 3 shows the total number of clones isolated and frozen down for each fusion and how they reacted in both the ELISA and Western blot assays. The serovar L_2 fusion was interesting as the clones were screened by ELISA and yet we ended up with three clones which were ELISA negative. In the serovar G fusion, the greater emphasis on MAbs to non-MOMP/non-LPS antigens gave an increased number of ELISA negative clones. Table 4 shows the antigens reacting with the Western blot positive MAbs and their ELISA assay status. All anti-MOMP and anti-LPS MAbs were ELISA positive, while the MAbs against the other antigens were ELISA negative except for that directed against the 29 Kd antigen from the first fusion. The MAb against this antigen from the third fusion was ELISA negative. Table 5 shows the Western blot results using the supernatants from 113 arbitrarily picked wells at the initial screening. Only 27 wells were positive overall with one-half of these having anti-MOMP antibodies. Interestingly, the next most prominent antibodies were those directed against the 57 Kd antigen. In three cases, there were two different antibodies in one well. From all of the hybridomas isolated, we focused our attention on 13 which are listed in Table 6. A preliminary characterization of each MAb is also listed.

TABLE 3. ELISA[1] and Western blot[1] results for the MAbs isolated in the hybridoma experiments.

Immunizing Serovar	Total No. Hybridomas Isolated	ELISA					
		Positive			Negative		
			Western Blot			Western Blot	
		No.	Pos.	Neg.	No.	Pos.	Neg.
L_2	42	39	20	19	3	2	1
J	26	24	6	18	2	2	0
G	31	21	4	17	10	7	3

[1] The MAb were tested using the homologous serovar as the test antigen.

TABLE 4. Western blot antigen specificities for the MAbs isolated in the three hybridoma experiments.

Antigen	Immunizing Serovar		
	L_2 (22)[a]	J (8)[a]	G (11)[a]
40 Kd (MOMP)[b]	11	6	2
10 Kd (LPS)[b]	8	-	2
75 Kd[c]	-	1	-
70 Kd[c]	-	-	2
60 Kd[c]	-	1	-
57 Kd[c]	-	-	4
32 Kd[c]	2	-	-
29 Kd	1[b]	-	1[c]

a Number of MAbs which were Western blot positive.

b ELISA positive.

c ELISA negative.

TABLE 5. Western blot antigen specificities for 113 wells which contained hybridoma and were screened in the serovar G experiment[1].

Antigen	No. of Wells Positive by Western Blottting
40 Kd (MOMP)	13
57 Kd	7
60 Kd	2
100 Kd	2
95 Kd	1
90 Kd	1
55 Kd	1
30 Kd	1
29 Kd	1
10 Kd (LPS)	1

[1] Twenty-seven (24%) wells produced 30 different MAbs to 10 different antigen of C.trachomatis were detectable by Western blotting.

TABLE 6. Characterization of monoclonal antibodies according to isotype, macromolecular specificity, antigenic specificity and surface exposure.

Monoclonal Designation	IgG Isotype	Epitope Recognized	Immunoblot Reaction	Micro-IF Reaction	ELISA
UM-1	1	MOMP	L2,B,E,D	L2,B,E,D,G,MoPn	+
UM-2	2a	MOMP	G,F,C	G,F,C,K	+
UM-3	1	MOMP	L2,E,G,MoPn	L2,E,G,MoPn	+
UM-4	2b	MOMP	L2	L2	+
UM-5	1	MOMP	J,H,I,A,C	J,H,I,A,C	+
UM-6	2a	MOMP	A-K,L2,MoPn,Cp	C,J,I,A	+
UM-7	2b	10Kd	A-K,L2,MoPn,Cp	A-K,L2,MoPN,Cp	+
UM-8	2a	29Kd	A-K,L2	-	+
UM-9	2b	32Kd	A-K,L2	-	-
UM-10	1	57Kd	A-K,L2	-	-
UM-11	2a	60Kd	A-K,L2,MoPn	-	-
UM-12	1	70Kd	A-K,L2,Cp	-	-
UM-13	1	75Kd	A-K,L2,MoPn,Cp	-	-

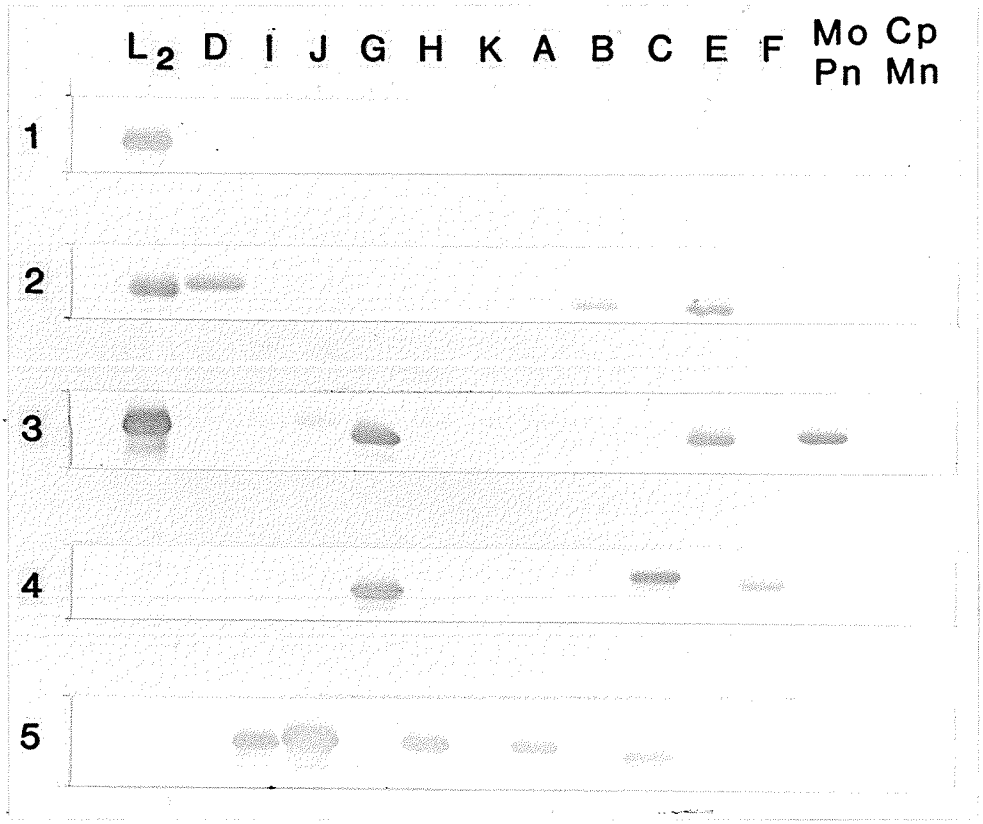
5. Characterization of Thirteen Monoclonal Antibodies Against Eight Different Antigens of C.trachomatis

All of the clones produced from the three fusions were initially characterized by Western blotting, ELISA and MIF assays. From this large group of MAbs, 13 were chosen for further study. They are listed in Table 6. There are six MAbs recognizing the MOMP and seven MAbs recognizing nonMOMP antigens. These MAbs were immunoblotted individually against an entire panel of C.trachomatis serovars, the MoPn strains and the C.psittaci Mn strain. The MIF assay was also carried out using these same strains while the ELISA was done using only the serovar from which the MAb was derived.

The MOMP MAbs will be discussed first. Only one, UM-4, was type specific. It reacts with an epitope found on the MOMP of serovar L_2. This was confirmed by the MIF assay as well. Four MAbs, UM-1, UM-2, UM-3 and UM-5, were subspecies specific and reacted with epitopes which were common to some but not all of the MOMPs from various serovars. There was some minor discordance in three of these MAbs between the Western blot reaction and that seen with the MIF assay. In two cases, the MIF reaction recognized other serovars not seen by the Western blot method and in one case, the Western blot reaction recognized a larger number of serovars than the MIF reaction. The other two MOMP MAbs had identical reactions between the two assay methods. There was one genus specific MAb reacting with the MOMP. On the Western blot assay, the strength of the reaction was substantially less than that seen with the other MOMP MAbs but was still greater than negative controls. However, the MIF assay only picked up four serovars. The Western blot reactions using the MOMP MAbs is seen in Figure 8.

Seven non-MOMP MAbs were chosen for further study. A MAb against a 10 Kd antigen was genus specific leading us to believe that it recognized the

FIGURE 8. Immunoblots developed with MAb (diluted 1:100) against the MOMP of the protein profiles seen in Figure 1. The chlamydial strains are listed across the top and the individual MAb used are listed on the left. Lane 1: UM-4 (L_2); lane 2: UM-1 (L_2,D,B,E); lane 3: UM-3 (L_2,G,E,MoPn); lane 4: UM-2 (G,C,F); lane 5: UM-5 (I,J,H,A,C).



LPS of chlamydia. Our gel system, using 10% or 12% PAG, would not allow us to give a better estimate of the MW other than 10 Kd.

The 29 Kd MAb (from the serovar L_2 fusion) was subspecies specific in that it reacted with all C.trachomatis serovars but not the MoPn strain. Some authors would term this species specific. The MW of this antigen was common to all serovars with no deviation especially among the serovars A, B and C. This MAb was the only non-MOMP, non-LPS MAb to be positive in the ELISA assay. The intensity of the colour reaction was about one-half of that produced with a MOMP or LPS MAb. The MIF reaction, however, was negative. The 32 Kd MAb was also subspecies specific again not reacting with any antigen in MoPn. This MAb explained the minor banding differences at about 32 K MW seen when immunoblots were done using human sera. The epitope on the 32 Kd antigen in serovar L_2 is found on an antigen which has a slightly lower MW in all of the other serovars.

While we isolated three separate clones recognizing the 57 Kd antigen, we chose to work with only one. Initially, all three showed the same blotting patterns and there was no attempt made to determine if these three were recognizing the same or different epitopes. This MAb also reacted with all serovars. The blotting reaction followed that seen on silver stain of PAG in that the region of the 57 Kd antigen was unfocused and did not form a discreet band as seen with the antigens both above and below it. This MAb was also negative by MIF and ELISA.

The 60 Kd MAb was truly species specific as it reacts with all serovars and the MoPn strain. There was no variation in MW among the serovars, however, the epitope is found on an antigen of slightly higher MW in MoPn. Again, there were negative results using the MIF and ELISA assay.

The MAb which recognizes the epitope found on a 70 Kd antigen was quite interesting. It could be considered genus specific as it reacts with

C.psittaci Mn strain and all the C.trachomatis serovars, however, there was no reaction with the MoPn strain. The epitope in CpMn is on a slightly higher MW antigen than that seen in the C.trachomatis serovars which showed no MW variation. Again, there was no reaction when the ELISA and MIF assays were used.

The final MAb to be discussed reacts with an epitope which is truly genus specific. There is a common MW antigen (75 Kd) in the C.trachomatis strains but a slight upwards deviation with the CpMn strain. This suggests there may be some relationship between the 75 Kd and 70 Kd antigens, as they show the same type of MW gain in CpMn, however the two corresponding MAb's are definitely different. The ELISA and MIF assays were negative.

The Western blot reaction of the MAbs against the antigens of MW 75 K, 70 Kd, 60 Kd, 57 Kd, 32 Kd and 29 Kd from the serovars L_2 and B and the strains MoPn and CpMn are shown in Figure 9.

6. Solubilization of Chlamydial Antigens with Detergents and a Reducing Agent

Caldwell et al (1981) has previously shown that MOMP is insoluble when whole EBs are suspended in sarcosyl but is readily solubilized when SDS is used to resuspend the sarcosyl insoluble material. In this way, he was able to produce what was termed the complete outer membrane complex (COMC) which is heavily enriched in MOMP. We used essentially the same procedure to divide the proteins of whole EBs into three different fractions.

Following one hour of incubation at 37°C in 2% sarcosyl and a high speed centrifugation, two fractions are resolved. The supernatant contains the sarcosyl soluble portion of the EB (Figure 10, lane B). From the silver stained gel, this fraction appears essentially the same as the whole EB (Figure 10, lane A) except the MOMP is missing. Also missing is the

FIGURE 9. Polyacrylamide gel (10%) stained with silver (on the left) and individual immunoblots (on the right) of the same gel developed with six different MAbs. The MW of the antigen with which each MAb is reactive is listed to the right of the immunoblots. MW estimates for the silver stained gel are given on the left with the C.trachomatis serovars L_2 and B, the mouse pneumonitis (MoPn) strain and the C.psittaci (CpMn) strain listed across the top.

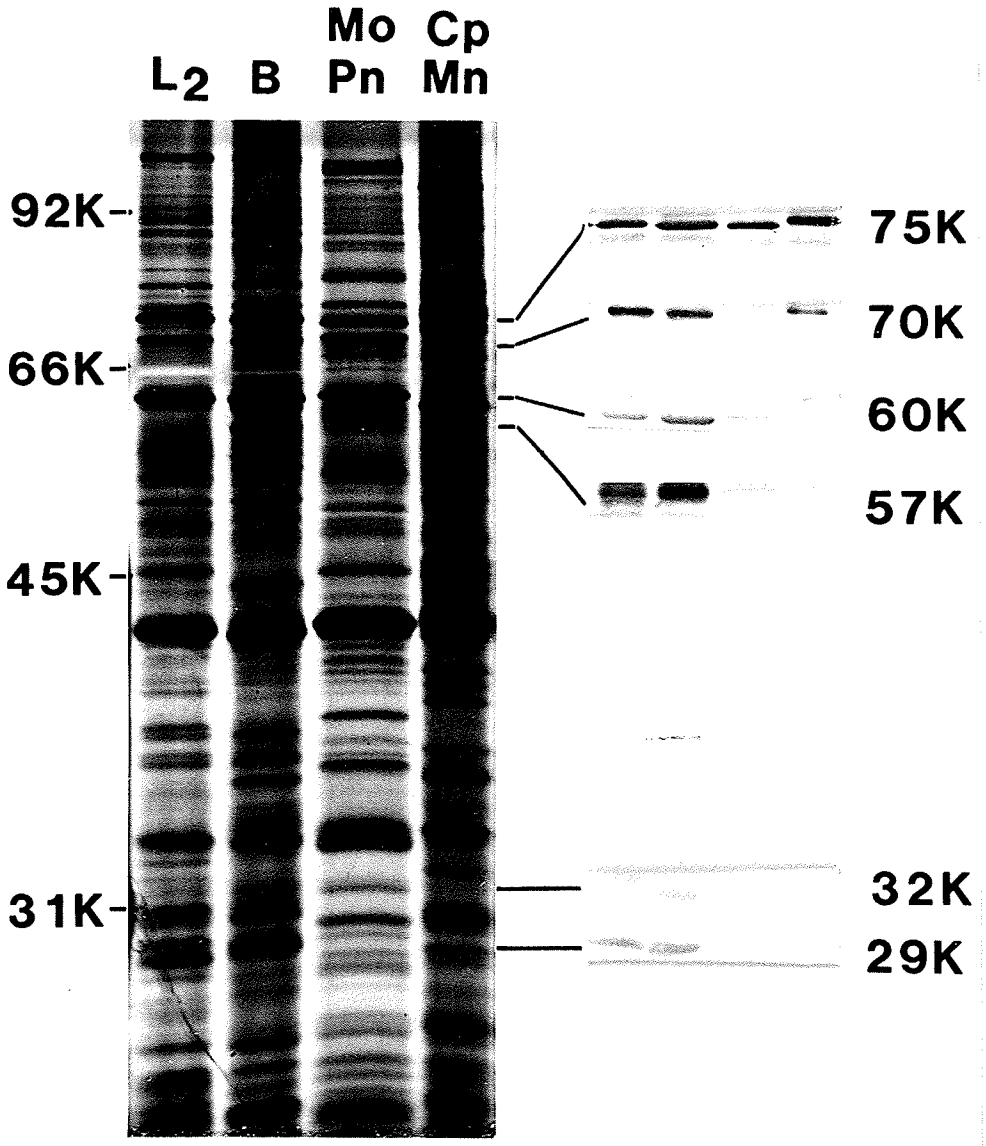
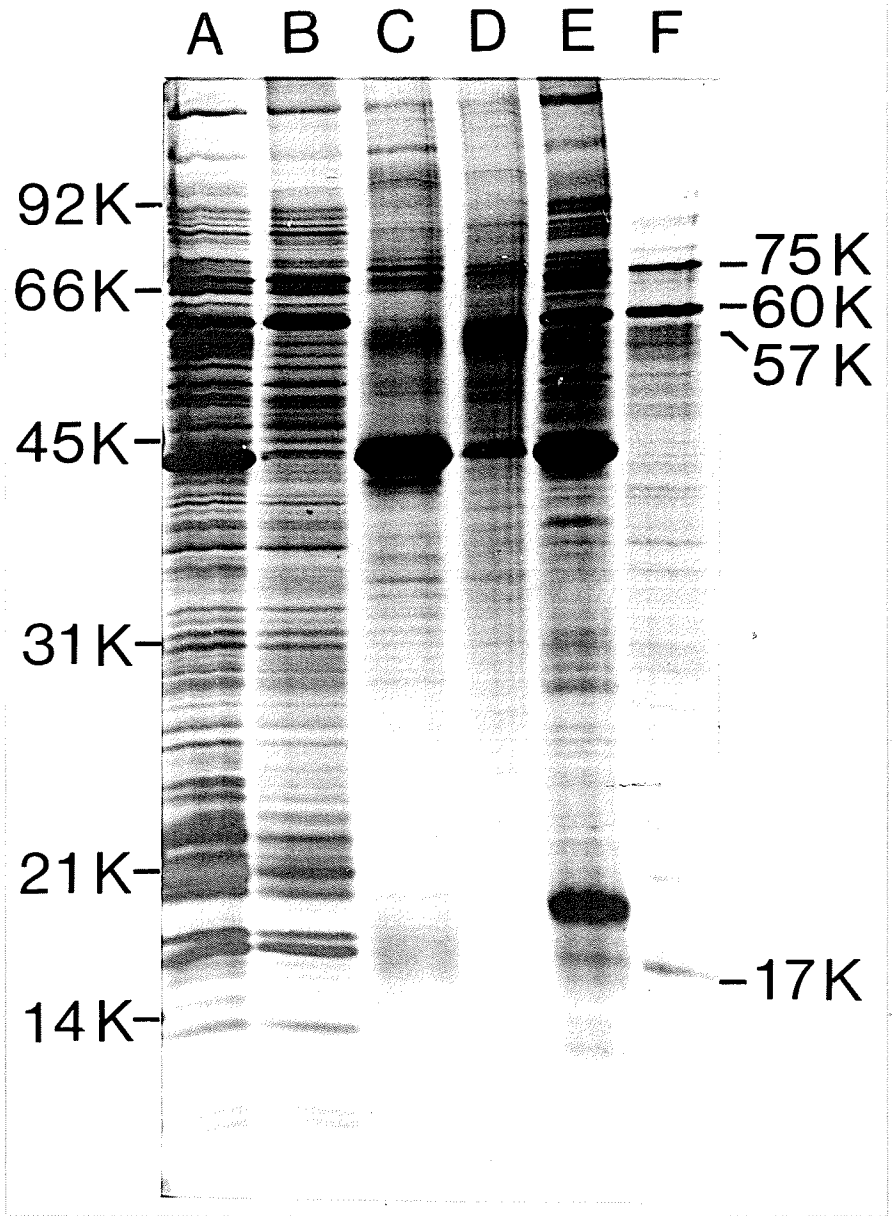


FIGURE 10. A 12% PAG silver stained of whole EB serovar L2 treated with detergents or dithiotreitol (DTT). Lane A: whole serovar L2 EBs; lane B: 2% sarcosyl soluble supernatant; lane C: sarcosyl insoluble pellet; lane D: 2% SDS soluble supernatant of pellet in lane D; lane E: 20 mM DTT insoluble antigens; lane F: 20 mM DTT soluble antigen. The molecular weights are listed on the left and the specific antigens are listed on the right.



protein at approximately 57 Kd. When the sarcosyl insoluble pellet is resuspended in SDS and incubated in the temperature range of 37°C-60°C, the majority of the MOMP is solubilized. After high speed centrifugation of the SDS suspension, another two fractions arise. The SDS soluble portion (Figure 10, lane C) contains basically MOMP and some 57 Kd antigen with other high MW proteins seen as a minority. The SDS insoluble pellet (Figure 10, lane D) is heavily enriched in the 57 kd antigen and contains a small amount of MOMP. As the temperature of incubation goes from 37°C to 60°C, more MOMP is solubilized and moves from the SDS insoluble pellet into the SDS soluble fraction.

The antigens of these three different fractions were separated on PAG and transferred to NCM and then probed with the various MAbs. Table 7 outlines the reactivity of the MAbs to each of the fractions. It shows that the 29 Kd, 32 Kd and 60 Kd antigens are entirely sarcosyl soluble. The 70 Kd and 75 Kd antigens are essentially sarcosyl soluble with a minor amount (about 10%) remaining to be solubilized by SDS. The LPS is divided evenly between the sarcosyl soluble and SDS soluble fractions. The MOMP itself is insoluble in sarcosyl as is the 57 Kd antigen. At 37°C, about one-half of the MOMP is solubilized in SDS while none of the 57 Kd antigen is found in this fraction. If 60°C is used instead, most of the MOMP is found in the SDS soluble fraction with some 57 Kd antigen leaving only a small amount of MOMP and the majority of the 57 Kd antigen in the SDS insoluble fraction. At 37°C, all of the 57 Kd antigen is in the SDS insoluble portion. The method used to solubilize the SDS insoluble pellet was to add reducing agents.

Disulfide bonding is known to play an important part in the outer membrane stability of a chlamydia. As well, our data seemed to suggest that the MOMP and the 57 Kd antigen might be linked by disulfide bonds. We

TABLE 7. Reactivity of eight MAbs with chlamydial antigens in three different detergent fractions.

MAb	Detergent Fraction		
	Sarcosyl Soluble	Sarcosyl Insoluble but SDS Soluble	Sarcosyl Insoluble and SDS Insoluble
1. 29	++++	-	-
2. 32	++++	-	-
3. MOMP	-	+++	+
4. 57	-	-	++++
5. 60	++++	-	-
6. 70	+++	+	+
7. 75	++	+	+
8. LPS	++	++	-

therefore looked at the effect of the treatment of whole EBs with dithiothreitol (DTT) alone with no detergents present. It was found that a number of antigens were released in significant amounts. After 30 minutes of suspension in 10 mM DTT, the whole EBs were spun down and the supernatant was saved and the pellet resuspended in buffer. The protein profile of the resuspended EBs (Figure 10, lane E) is essentially the same as untreated EBs (Figure 10, lane A). However, the DTT soluble supernatant (Figure 10, lane F) shows that some proteins have been released. Specifically, the 75 Kd, 60 Kd and 57 Kd proteins. There was also a substantial amount of a 17 Kd protein which we had previously not seen before reacting with any of the human sera immunoblots or with sera from immunized mice.

These solubilized proteins from serovars L2 and D were used to immunize mice in order to raise polyclonal sera which would not have any anti-MOMP antibody. The serovar D antigens raised antibody against the 57 Kd antigen only while the antigens from serovar L2 raised antibodies against both the 17 Kd and 57 kd antigens. When these sera were used in immunoblots, which included the MoPn and C.psittaci Mn strains, a number of interesting results were seen. The antisera from both the serovar L2 and D immunizations reacted with the 57 Kd antigen in the C.trachomatis serovars but an antigen of slightly higher MW was recognized in the MoPn strain while no corresponding antigen was seen with CpMn (Figure 11). There is a faint suggestion of a comparable antigen being present in CpMn at that MW range but nothing definite. Examination of the silver stained gel (Figure 1) with whole EBs shows antigen just below the 60 Kd antigen in the MoPn lane which could represent this 57 Kd-like antigen.

Two separate sets of sera were obtained from mice immunized with the DTT soluble antigens from serovar L2. In one case, the sera recognized a species specific 17 Kd antigen (Figure 12a). The MW was seen to vary con-

FIGURE 11. Immunoblot using polyclonal sera (1:250) from mice immunized with antigen solubilized with DTT from serovar D or L2 (both immunoblots were the same). The chlamydial antigens are whole EBs from C.trachomatis serovars L2, D, B and the strain mouse pneumonitis and the C.psittaci strain meningopneumonitis.

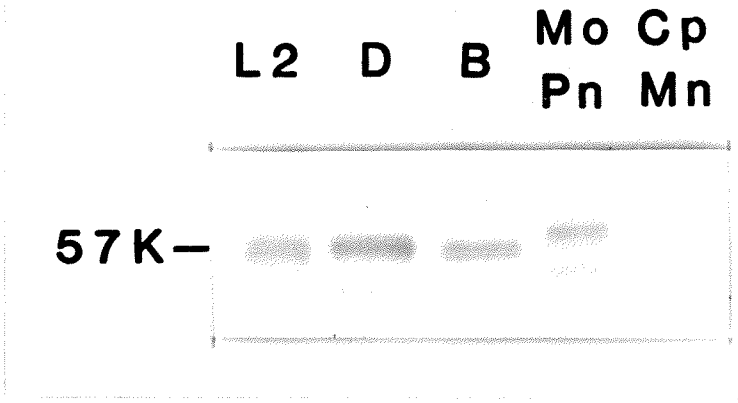
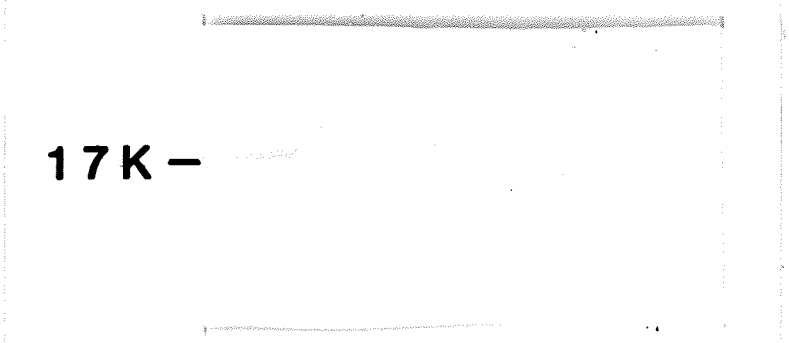
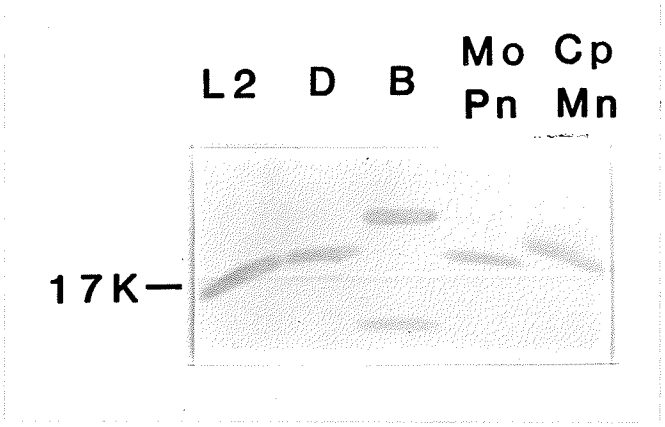


FIGURE 12. Immunoblots using two different sets of sera (1:250) from mice immunized with the dithiothreitol (DTT) soluble antigens from serovar L2. The chlamydial antigens are whole EBs from C.trachomatis serovars L2, D and B, and the strain mouse pneumonitis and the C.psittaci strain Mn.

A.



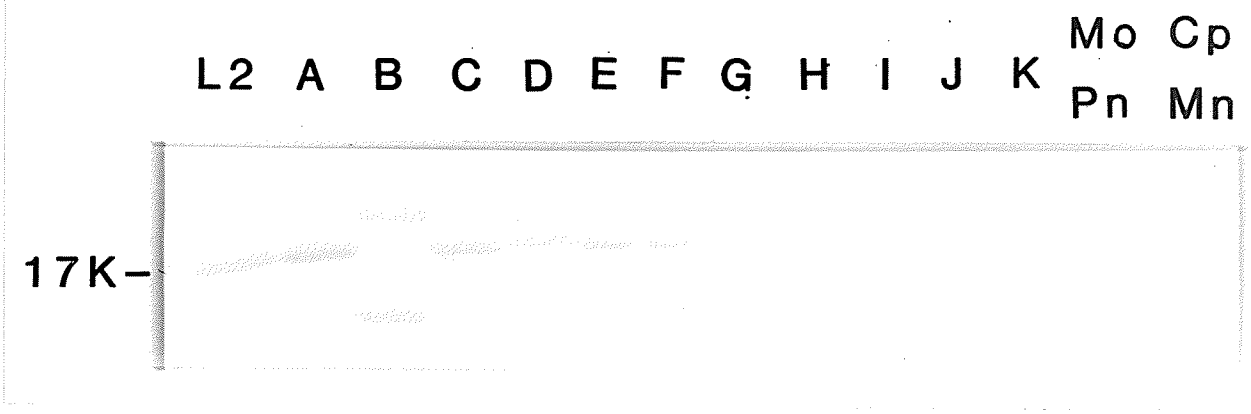
B.



siderably with a slightly smaller antigen being recognized in serovar D and an antigen of approximately 15 Kd being recognized in serovar B. The MoPn strain has a band at about 17 Kd while no reaction is seen with the CpMn strain. The second set of antisera gave a different result. The same 17 Kd antigen was seen with serovar L2 but now an antigen of equal MW was recognized in serovar D with the lower MW antigen only slightly seen (Figure 12b). The same antigen is seen in MoPn but two new antigen bands have also appeared. One is an approximately 18 kd antigen in serovar B which is accompanied by the 15 Kd antigen seen previously and there is now an antigenic band at 17 Kd in the CpMn lane. It would appear that there are two antigens at 17 Kd MW, one which is species specific and the other is genus specific. When the sera which recognized the genus specific epitopes is used on all the chlamydial strains in an immunoblot, a number of patterns are seen (Figure 13). Serovars L2, A, C, E and F appear to have only one band. Two antigenic bands (with similar MW) are seen in serovars D, G, H, I, J and K, while serovar B has two widely separated antigens. The MoPn strain has only one band which might be classed with the L2, A, C, E and F group while the CpMn strain also has only one major band. Knowing where to look allows us to review the silver stained gel (Figure 1) and see this 17 Kd antigen with its major and minor MW variations. The serovars L2, A, C, E and F all appear to have a major and minor band which on the blot appears as a single band. The other serovars have two bands of equal amounts which show up on the blots as two separate and distinct antigens.

Overall, these sera were interesting as only the 57 Kd and 17 Kd antigens raised an immune response. The 60 Kd and 75 Kd antigens were well represented among the DTT soluble antigens and yet, no antibody response against them was seen on the immunoblot. When these sera were tested in an ELISA assay, using serovars L2, D and B, it was negative, suggesting that

FIGURE 13. Immunoblot using sera (1:250) from mice immunized with antigens solubilized with DTT from serovar L2. The chlamydial strains are listed across the top and the MW of the genus specific antigen the sera reacted with is listed at the left.



the 57 Kd and 17 Kd antigen epitopes are not surface exposed. The 17 Kd antigen is also found in the sarcosyl soluble fraction from whole EBs.

7. Purification of Chlamydial Antigens Using Affinity Chromatography

All of the MAbs listed in Table 6, except UM-13 (anti-LPS), were linked to CNBr- activated sepharose. Detergent solubilized fractions of whole EB's from serovar L2 containing the appropriate antigen were incubated with the MAb-sepharose overnight at 4°C and any material which bound to the column was eluted with .1 M acetic acid/.15 M NaCl. A portion of each fraction collected was run on PAG to identify antigen containing fractions. These fractions were pooled and used to immunize mice in order to raise monospecific polyclonal sera.

The overall results were somewhat disappointing in that only MOMP, the 57 Kd and the 75 Kd antigens were purified. The other columns used to purify the 29 Kd, 32 Kd, 60 Kd and 70 Kd antigens did not appear to work as none of these antigens were seen in the eluted fractions. In some cases, the fractions were pooled blindly and injected into mice, but there was no antibody response to any of the antigens.

The three columns which gave positive results had good amounts of antigen in the eluted fractions. These were pooled and used to immunize mice over a four week immunization schedule. The sera were used in immunoblots, ELISA assays and neutralization experiments.

The results are seen in Table 8. The sera against the 75 Kd antigen were genus specific in the immunoblot as seen in Figure 14. The ELISA assay using whole EBs from serovar L2 as antigen was positive at a dilution of 1:1000. The MIF assay was negative at this dilution but was positive at a dilution of 1:25. There was neutralization of infection using this serum at a dilution of 1:10. Thus, this result suggests the 75 Kd antigen may play a role in the infection process.

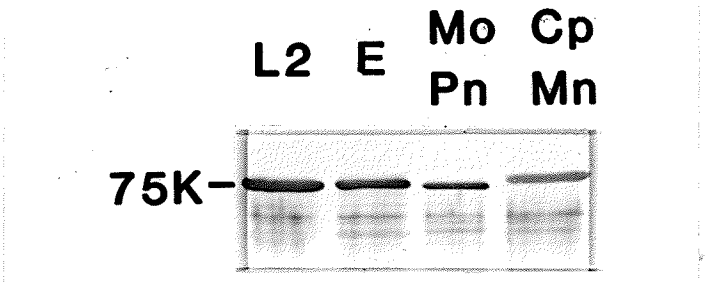
TABLE 8. Characteristics of monospecific polyclonal antibodies to chlamydial antigens.

Monospecific Antibodies to	Immunoblot Specificity	ELISA	Micro-IF	Neutralization titer* 1:10	1:100	1:1000
75 Kd	Genus	+	+	+†	-	-
57 Kd	Species	-	-	-	-	-
MOMP	Genus	+	+	+†	+	-

* Neutralization was defined as a greater than 50% reduction in IFU's with serovar L2.

† Neutralization was detectable without added guinea pig complement.

FIGURE 14. Immunoblot using monospecific polyclonal sera (1:250) from mice immunized with the L2 antigen purified from the 75 Kd MAb affinity column. The chlamydial strains are listed across the top and the antigen with which the sera reacted is listed at the left.



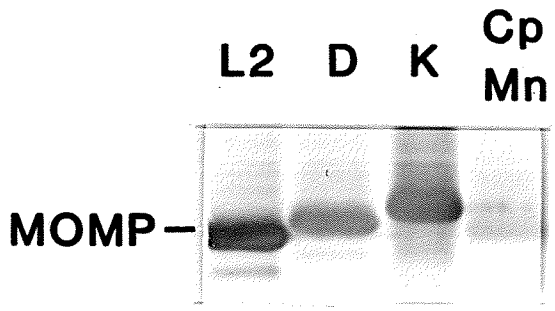
The serum raised by immunization with the 57 kd antigen was only species specific on the immunoblot (Figure 15). There was no reaction with any antigen of the CpMn strain. Although not shown, there was a similar reaction pattern with the same antigen in MoPn as seen previously in Figures 11a and 11b. Other than the immunoblot, this serum was non-reactive as it was negative when used in the ELISA, MIF and neutralization assays.

The anti-MOMP sera acted in a manner similar to sera against whole EBs. On immunoblot, it was genus specific (Figure 16) reacting very strongly with the C.trachomatis serovars and less strongly, but distinctly, against CpMn strain. The other assays were all positive with the neutralization assay being positive at 1:100 dilution as compared to the neutralization at only 1:10 dilution produced by the anti-75 Kd antigen sera.

In a different approach to the problem, polyclonal sera were raised against the 75 kd antigen which was the result of cloning the respective gene from serovar D into a pUC 18 vector (S. Danilition, M.Sc. Thesis, University of Manitoba, 1987). The resulting gene product was a 75 Kd protein with the majority of which was present in the cytoplasm of the host E.coli strain DH5-1. The cytoplasmic fraction was separated on a PAG and the 75 Kd protein electroeluted and used to immunize mice. The resulting polyclonal serum was genus specific on immunoblot when tested against serovars L2, B, D, G and the MoPn and CpMn strains. When used in an ELISA assay, it was positive at dilutions of 1:400, 1:1600 and 1:200 with whole EBs from serovars L2, B and the C.psittaci Mn strain, respectively. This is a strong indication that the 75 Kd protein is a surface exposed genus specific antigen. The polyclonal sera were not used in neutralization studies.

FIGURE 15. Immunoblot using sera (1:250) from mice immunized with the L2 antigen purified from the 57 Kd MAb affinity column. The chlamydial strains are listed across the top and the antigen with which the sera reacted is listed at the left.

FIGURE 16. Immunoblot using monospecific polyclonal sera (1:250) from mice immunized with the L2 antigen purified from the MOMP MAb affinity column. The chlamydial strains are listed across the top and the antigen with which the sera reacted is listed at the left.



8. Protease Treatment of Whole EBs

We undertook to determine the susceptibility of whole EBs of various serovars to the three proteases, V8 protease, trypsin and chymotrypsin. These enzymes cut at glutamic acid (V8 protease), lysine or arginine (trypsin) and phenylalanine, tryptophan or tyrosine (chymotrypsin). These experiments should yield additional information on the molecular conformation of native MOMP in the EB outer membrane. A small amount (1 ug) of each enzyme was allowed to incubate overnight with the whole EBs and the amount of proteolytic action determined using silver staining and Western blotting after the EB protein was separated on PAG. The gels were blotted with MOMP MAbs and polyclonal sera from mice immunized with the outer membrane complex of serovar L2. This polyclonal serum was species specific.

Of the three enzymes, V8 protease was by far the least reactive. In the case of the serovars B, C and D, there was little, if any, cleavage of MOMP as visualized by the appearance of bands <40 Kd when blotted with the appropriate MOMP MAb. Other serovars (A, E, F, G, H, I, J, K and the MoPn strain) all had one band 3-4 Kd less than the corresponding MOMP which bound the MOMP MAb. Serovar L2 was the most susceptible to V8 protease, having a major band around 36 Kd but also some other proteolytic products between 36 Kd and 25 Kd. This initial information would suggest that for the majority of serovars, there is only one V8 protease site which is susceptible to proteolytic attack and that it appears to be the same distance from either the amino or carboxyl end of the MOMP giving the variable MW peptide which rises and falls with the initial MW of the MOMP. Overall, the enzyme is quite inactive as the majority of the MOMP remains intact after the incubation period. Examples of the immunoblots are seen in Figures 17 and 18. Figure 17 shows the serovars L2, D, B and E (both treated and untreated with V8 protease) blotted with the MOMP MAb UM-1.

FIGURE 17. Immunoblot developed with MOMP MAb UM-1. Whole EBs of the serovar listed across the top were treated or left untreated with V8 protease, then solubilized and separated on 12% PAG.

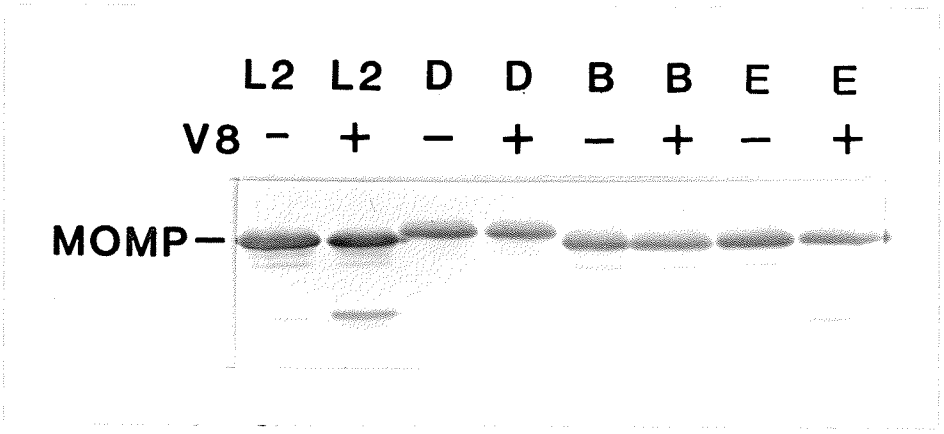
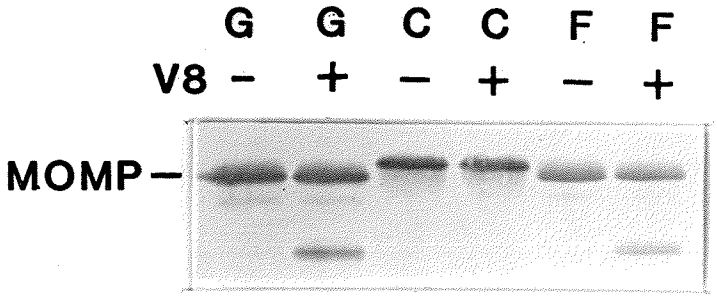


FIGURE 18. Immunoblot developed with MOMP MAb UM-2. Whole EBs of the serovar listed across the top were treated or left untreated with V8 protease, then solubilized and separated on 12% PAG.



Serovars G, C and F are blotted with the MOMP MAb UM-2 in Figure 18. Both figures show the 36 Kd peptide resulting from V8 protease cleavage of MOMP while with some serovars, no peptide is seen. Proteolytic cleavage may have resulted in a non-reactive epitope, however, the same result was seen when the polyclonal MOMP sera was used suggesting that this was not the case.

The enzyme digestions with trypsin and chymotrypsin were in complete contrast with those of V8 protease. After overnight digestion with trypsin and chymotrypsin, there was no whole MOMP left as it has all been broken down into smaller peptide fragments. An example of the immunoblot result is seen in Figure 19. The serovars L2, D, B and E were treated with trypsin and immunoblotted using the UM-1 MAb against MOMP. No intact MOMP is seen, with L2 showing two peptides at 25 Kd and 20 Kd and the other serovars having only one reactive peptide around 20 Kd. These results showed that trypsin and chymotrypsin are very active against the MOMP such that few of the resulting peptides had reactive epitopes.

We used serovars L2 and B treated with V8 protease, trypsin and chymotrypsin followed by immunoblotting to determine the fate of other chlamydial antigens. The idea was to look for different blotting patterns between treated and untreated profiles identifying which antigens were surface exposed such that they could interact with the proteases.

A summary of the results is given in Table 9, and an example of the immunoblots are seen in Figure 20. Each enzyme will be dealt with individually.

a) V8 Protease. Treatment of serovars L2 and B with V8 protease followed the previous results. The majority of the L2 MOMP was cleaved while the B MOMP was apparently untouched. The 75 Kd, 70 Kd and 60 Kd antigens of both

TABLE 9. Susceptibility of seven chlamydial antigens to protease digestion.

	Antigen[d]						
Protease/Serovar	MOMP	29 Kd	32 Kd	57 Kd	60 Kd	70 Kd	75 Kd
V8/L2	+[a]	+	++[b]	+	++	++	++
V8/B	-[c]	+	++	+	++	++	++
Trypsin/B	++	+	+	++	+	+	+
Chymotrypsin/B	++	+	+	++	+	+	++

[a] Residual uncleaved antigen remained.

[b] Complete cleavage of antigen.

[c] No apparent cleavage of antigen.

[d] Antigens were digested as part of the intact EB.

FIGURE 19. Immunoblot developed with the MOMP MAb UM-1. Whole EBs of the serovar listed across the top were treated or left untreated with trypsin and then solubilized and separated on 12% PAG. The MW of the peptides are listed on the left.

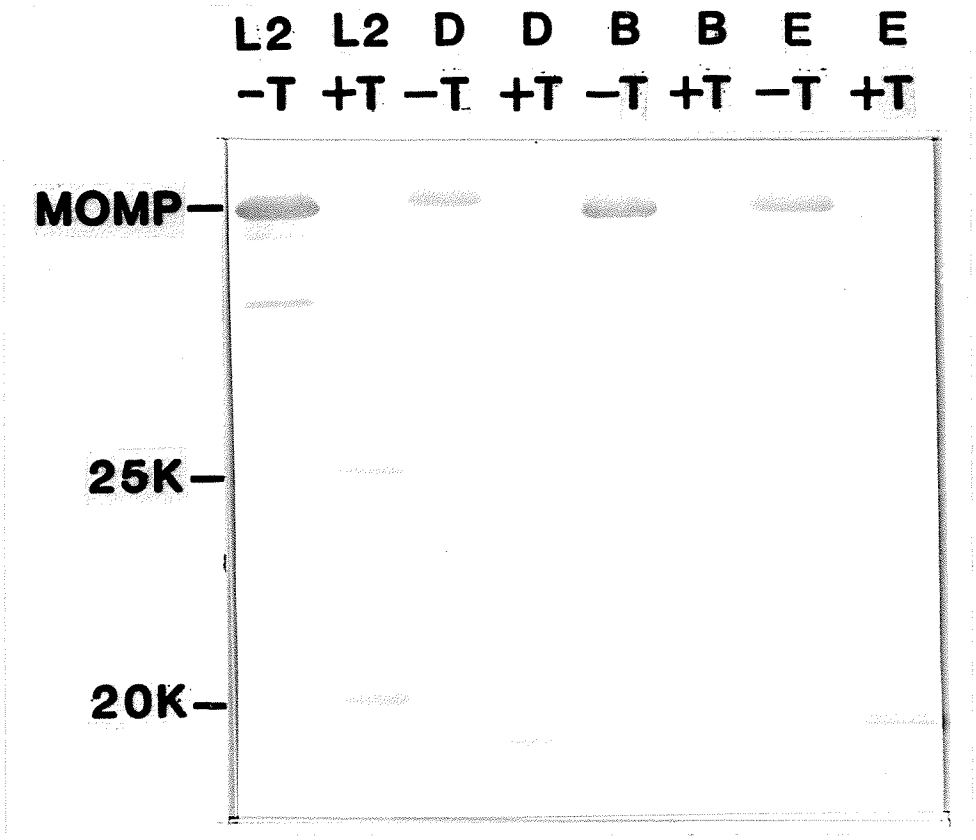
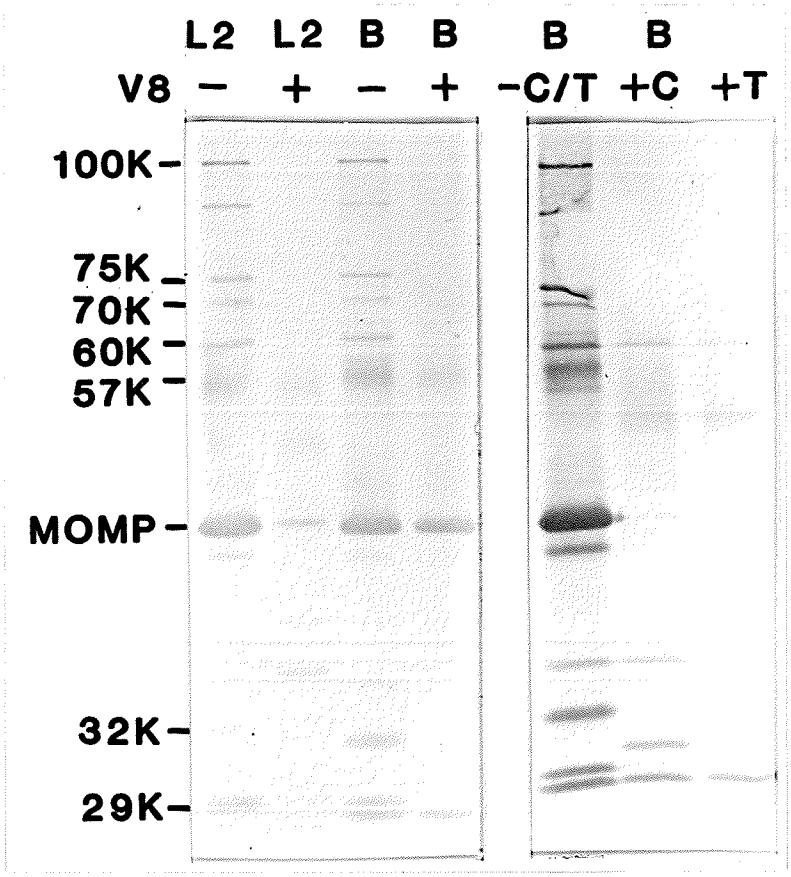


FIGURE 20. Immunoblot using sera (1:500) from mice immunized with serovar J. The EBs were left untreated or treated with either V8 protease (V8), trypsin (T), or chemotrypsin (C) and then solubilized and separated on 12% PAG. The serovars used are listed across the top with and without the protease and the MW of specific antigens of interest are listed on the left.



serovars were completely cleaved with no peptides seen at the lower MW when blotted with the individual MAb (data not shown). The 57 kd antigen was still present after treatment but in reduced amounts. The 32 Kd antigen showed complete cleavage again with no peptide fragments being seen on the blot. The 29 Kd antigen is perhaps the most interesting. The majority of this antigen is cleaved with no smaller peptides seen with the 29 MAb. However, the polyclonal sera (from the serovar G fusion) shows two bands at 29 kd with serovar B as previously seen in Figure 5. The upper protein which reacts with the 29 Kd MAb is cleaved while the lower band remains intact. This is seen only with serovar B. What antigen this protease resistant epitope is found on is unknown.

b) Trypsin and Chymotrypsin. These two enzymes gave essentially the same results with only minor differences. Only serovar B was used for these two enzymes. The MOMP was completely cleaved with a peptide seen at 30 Kd on blot. The 75 Kd antigen was also completely cleaved by chymotrypsin but with a small amount left after trypsin treatment. Residual amounts of the 70 Kd and 60 Kd antigens were left with both enzymes. The 57 Kd antigen was totally cleaved and gave a 50-55 Kd peptide which blotted with the 57 kd MAb showing that this epitope remained functional. The 32 Kd and 29 Kd antigens showed only a minor amount of antigen remaining after treatment. Again, as with the V8 protease, the lower antigen of the 29 kd doublet in serovar B was untouched by these two enzymes. It may not be surface exposed or have a susceptible site which is exposed to the surface.

9. Protease Digestion of ^{125}I-Labelled EBs

So far, we have shown that a number of antigens are susceptible to protease attack when found as part of the whole EB. This would suggest that they are surface exposed and accessible to the enzymes. To substan-

tiate these data, we radiolabelled whole EBs with ^{125}I and then treated them with either V8 protease or trypsin. This was done to see whether or not surface labelled antigens were cleaved and if we could identify these antigens and their digestion products using immunoblotting with MAbs and polyclonal sera.

The autoradiograph of the labelled EBs is seen in Figure 21. The undigested EBs show a large band at 40 Kd (MOMP) and a number of bands in the 60-70 Kd range. There is also another band at >100 Kd which is also quite prominent and a number of faint, but distinct, bands <40 Kd. We can line up the immunoblots and the autoradiograph directly as both labelled and unlabelled EBs were run on the same gel. The serovar G immune serum was used to develop the immunoblot in Figure 22. This serum reacts with a number of antigens which are radiolablled such as the MOMP and the antigens at >100 Kd, 75 Kd and 57-60 Kd. The other antigens at 29 Kd and 17 Kd may also be radiolabelled, but it is hard to say. A number of bands do line up with these antigens, but they are too faint to make a fair conclusion.

Digestion of the labelled and unlabelled EBs follows the same type of pattern as seen previously. The V8 protease has little effect on the MOMP but completely cleaves the 75 Kd and 60 Kd antigens (Figure 22). The 57 Kd antigen appears somewhat reduced but mainly intact. The 29 Kd and 17 Kd antigens are no longer present. One MW band, around 20 Kd, has appeared. When blotted with the MOMP MAbs, this new band is seen as a MOMP fragment (Figure 23).

The autoradiograph confirms that MOMP is intact and shows that the banding at the 75 Kd region is gone with decreased banding in the 60 Kd area. There is still some material left around 60 Kd which may be the 57 Kd antigen. A number of new bands are seen at less than 40 Kd which correlate exactly with the MOMP fragments when lined up with the MOMP MAb

FIGURE 21. Autoradiograph of whole serovar L_2 EBs extrinsically radiolabelled with ^{125}I, treated with a protease then separated on a 15% PAG. Lane A: untreated EBs; lane B: V8 protease; lane C: trypsin. The molecular weight estimates are listed on the left.

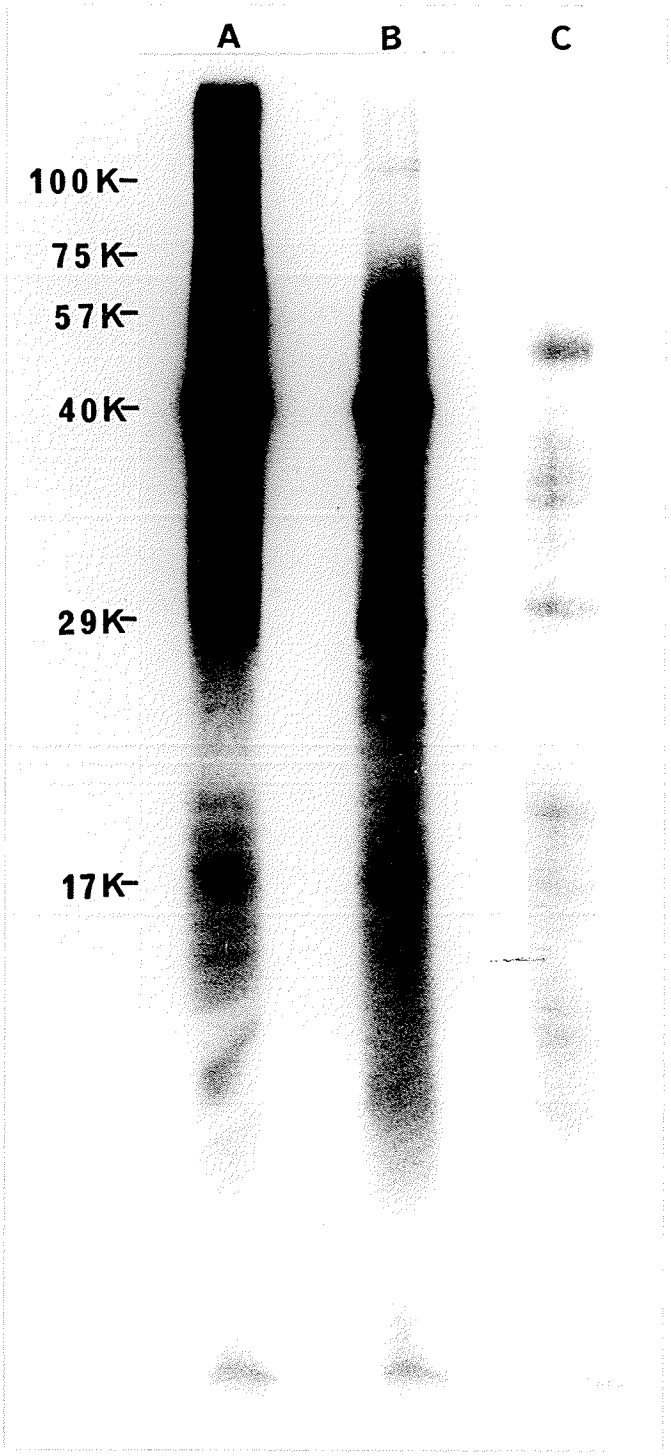


FIGURE 22. Immunoblot of whole serovar L_2 EBs treated with proteases, separated on a 15% PAG and developed with 1:500 dilution serovar G mouse sera. Lanes A, C and E are unlabelled while lanes B, D and F are extrinsically radiolabelled with ^{125}I. Lanes A,B: untreated; lances C,D: V8 protease; lanes E,F: trypsin. The molecular weight estimates are listed on the left. This immunoblot was used to produce the autoradiograph in Figure 21.

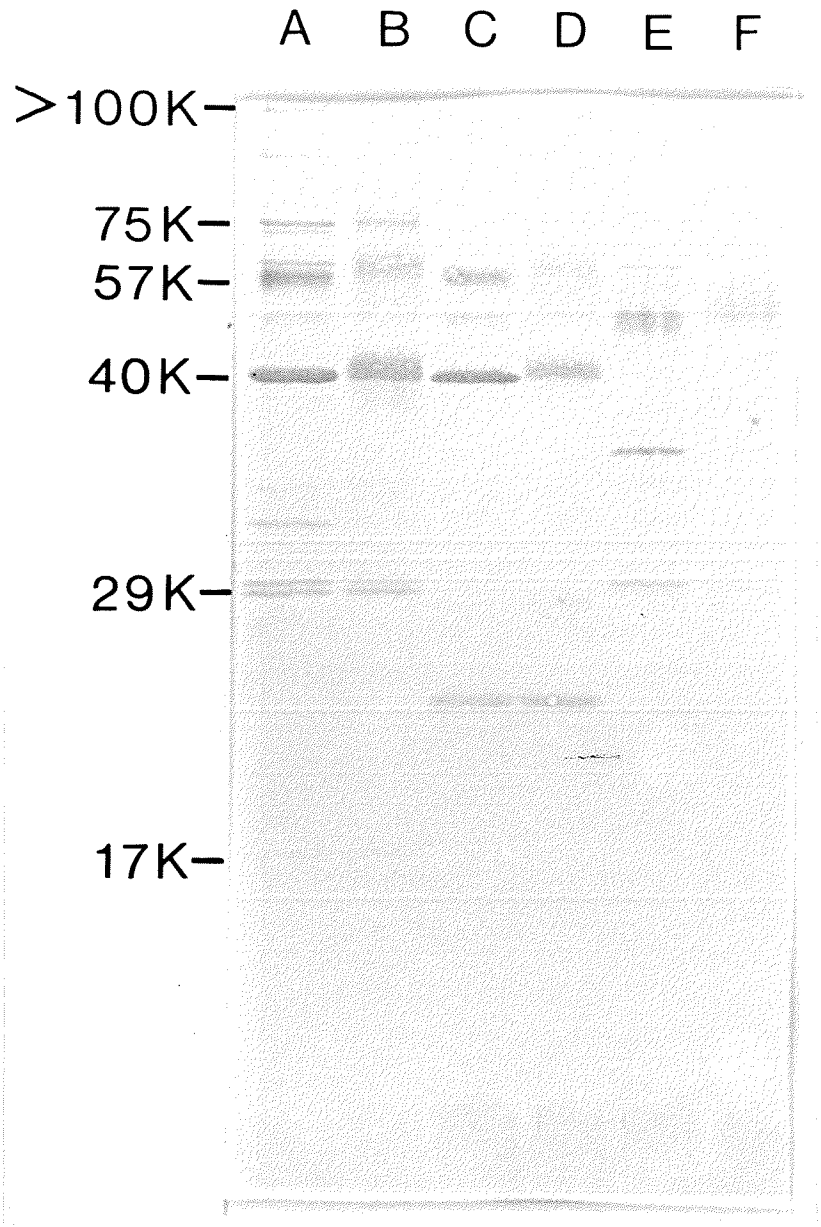
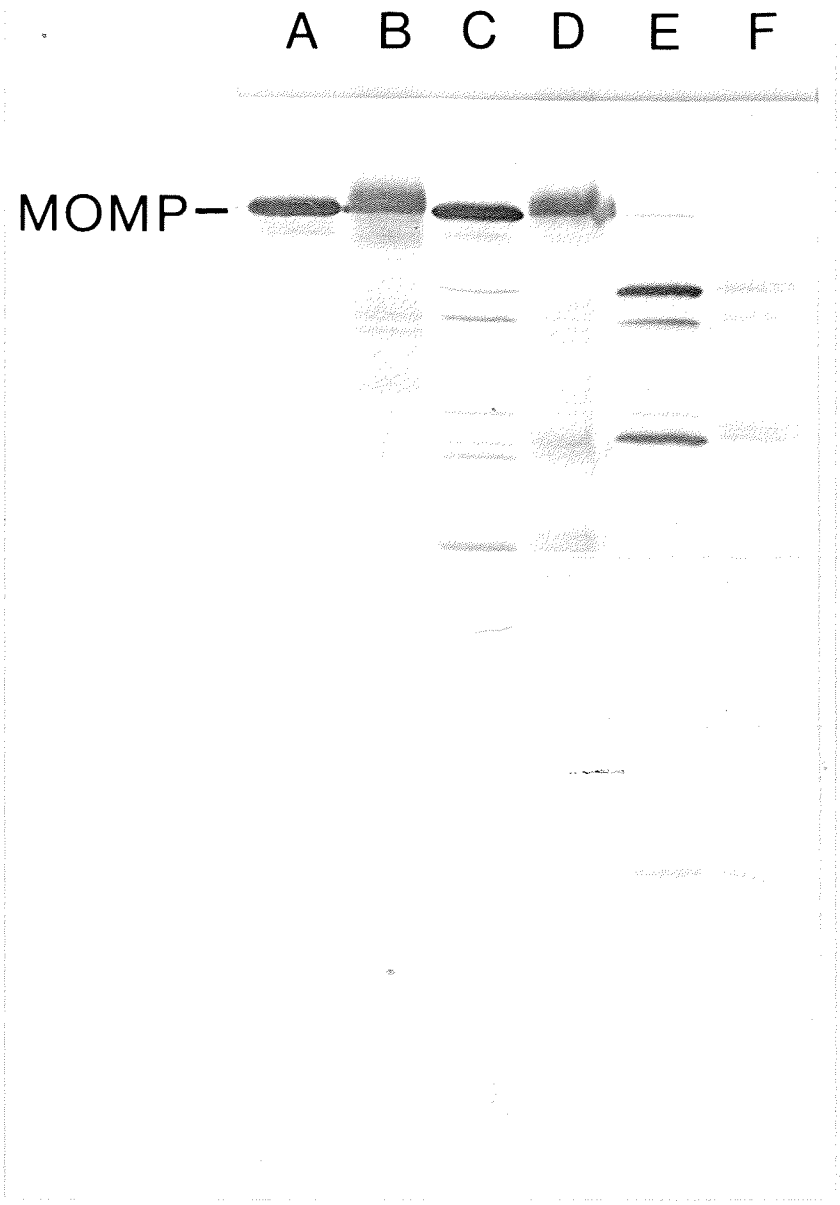


FIGURE 23. Immunoblot of whole serovar L_2 EBs treated with protease, separated on a 15% PAG and then developed with MOMP MAbs UM-3 and UM-4. Lanes A, C and E are unlabelled while lanes B, D and F are extrinsically labelled with ^{125}I. Lanes A,B: untreated controls; lanes C,D: V8 protease; lanes E,F: trypsin. This immunoblot produced an autoradiograph similar to that in Figure 21.



immunoblot. It is interesting in that the serovar G sera only picks up one MOMP fragment while the MOMP MAbs see six different peptides. The immunoblot using sera specific for the 17 Kd antigen (data not shown) shows that it is also digested by the V8 protease. There is a band on the autoradiograph which may represent the 17 Kd antigen and appears to fade after V8 protease treatment, but again, it is hard to come to a definite conclusion.

The trypsin digestion immunoblotted with the serovar G sera shows essentially complete digestion of the MOMP, the 75 Kd and 57 Kd antigens. There is a small amount of the 60 Kd antigen remaining. The 29 Kd and 17 Kd appear to be cleaved, although there is a band present around 29 Kd with another MW band present around 35 Kd. The MOMP MAb immunoblot shows these two bands to be MOMP fragments. These MOMP fragments again line up with MW banding patterns seen on the autoradiograph. The serovar G immunoblot also shows a MW band at 50-55 Kd which, from previous work using MAbs, should be the breakdown product from the 57 Kd antigen. The autoradiograph has a new band in the same spot suggesting that the 57 Kd antigen was radiolabelled and is cleaved by the trypsin. It is interesting that while appearing to be surface exposed, our 57 Kd MAb and polyclonal sera did not give a positive result in an ELISA assay using whole EBs.

The trypsin digest on the autoradiograph confirms the cleavage of the 75 Kd and 60 Kd antigens. The 29 Kd antigen is hard to judge as a newly produced MOMP fragment sits right in that area. The 17 Kd antigen shows a more definite disappearance with the trypsin digestion suggesting that it is surface exposed.

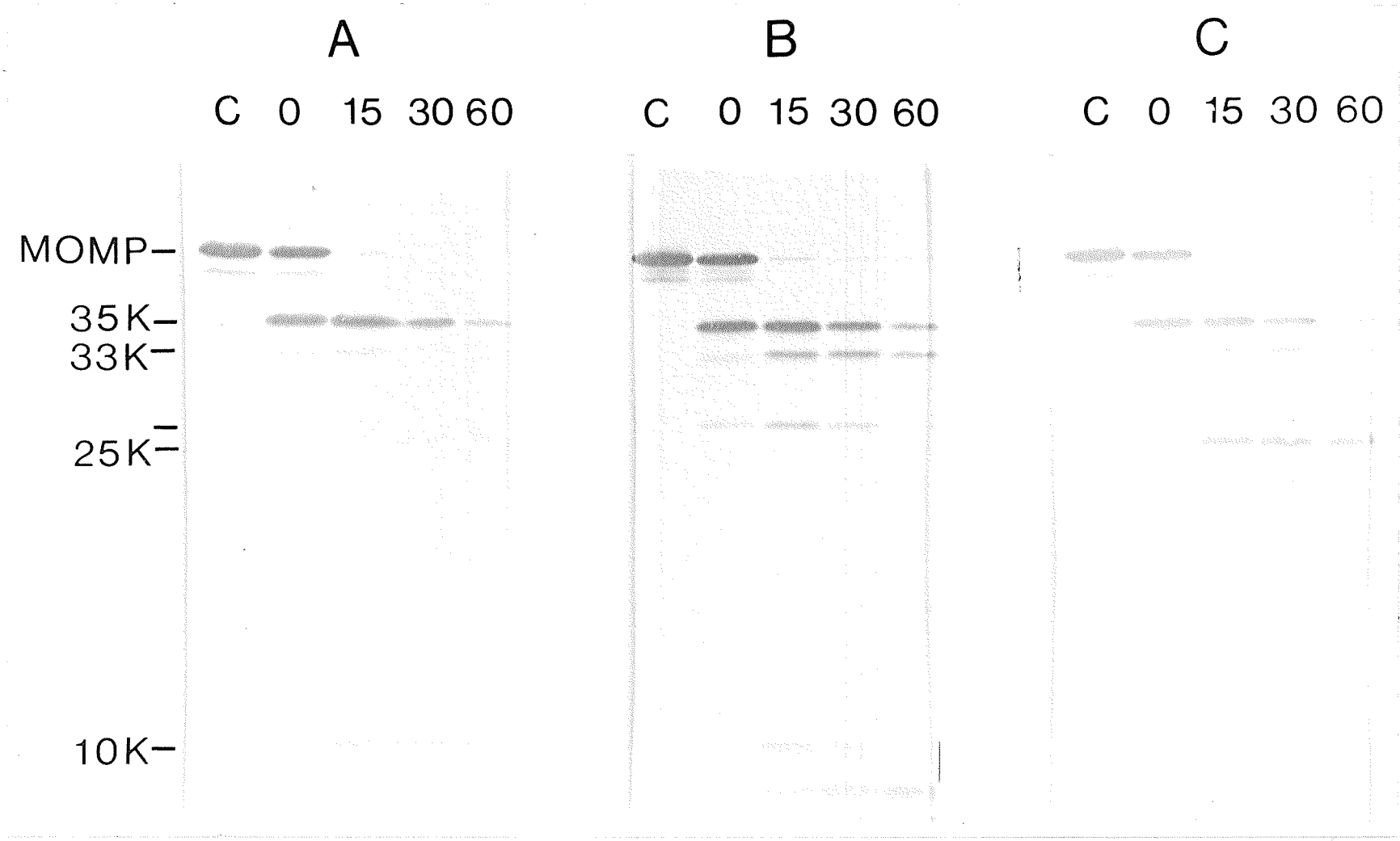
10. Sequential Digestion of Whole EBs with Trypsin

We treated whole EBs of various serovars with trypsin for different time periods, then separated the EB proteins by PAGE and immunoblotted with

MAbs against the MOMP. An example of this is seen in Figure 24. Whole EBs from serovar L2 were treated with trypsin for up to 60 minutes and the fate of the MOMP followed using the MAbs UM-1, UM-3 and UM-4. The 0 time period represents adding the trypsin and removing a sample immediately. Even within that short time space, there is substantial cleavage of the MOMP. In this figure, in part A, the blot was developed with UM-1, a subspecies MAb recognizing epitopes on serovars L2, B, D and E. It can be seen that trypsin cleaves the MOMP, producing a major peptide at about 35 Kd which retains the UM-1 epitope and a much smaller fragment around 10 Kd which also appears to bind the UM-1 MAb. However, by 60 minutes, very little of this binding ability remains for either fragment. A different pattern is seen with the subspecies MAb UM-3 (Figure 24, part B) which recognizes serovars L2, B, G and MoPn. It binds to an epitope on the same 35 Kd fragment as UM-1, but also to epitopes on 33 Kd and 25 Kd fragments. There are also two small fragments at 10 Kd which bind this MAb, one of which is the same one which has the UM-1 epitope. In part C of Figure 24, the blot has been developed with the MOMP MAb UM-4 which recognizes only serovar L2. Again, the same 35 Kd peptide fragment is initially seen followed by the lesser 33 Kd fragment, but now a new fragment at 24 Kd is seen. Only the type specific MAb appears to react with this fragment which persists as the trypsinization continues while the other fragments show continued breakdown.

All these data would suggest there is a specific sequence in which trypsin attacks the MOMP of serovar L2 on an intact EB. There is an easily accessible trypsin site which is quickly cleaved resulting in 35 Kd and a 5 Kd fragments (based upon a 40 Kd intact MOMP). This 35 Kd fragment has the three epitopes previously described. The initial cleavage allows access to a number of secondary sites resulting in new fragments of 33 Kd, 25 Kd and

FIGURE 24. Immunoblots of whole serovar L_2 EBs treated with trypsin for varying time periods, separated on a 12% PAG and developed with MOMP MAb UM-1 (A), UM-3 (B), and UM-4 (C). The time periods in minutes are given across the top (C is untreated control) and molecular weight estimates are listed on the left.

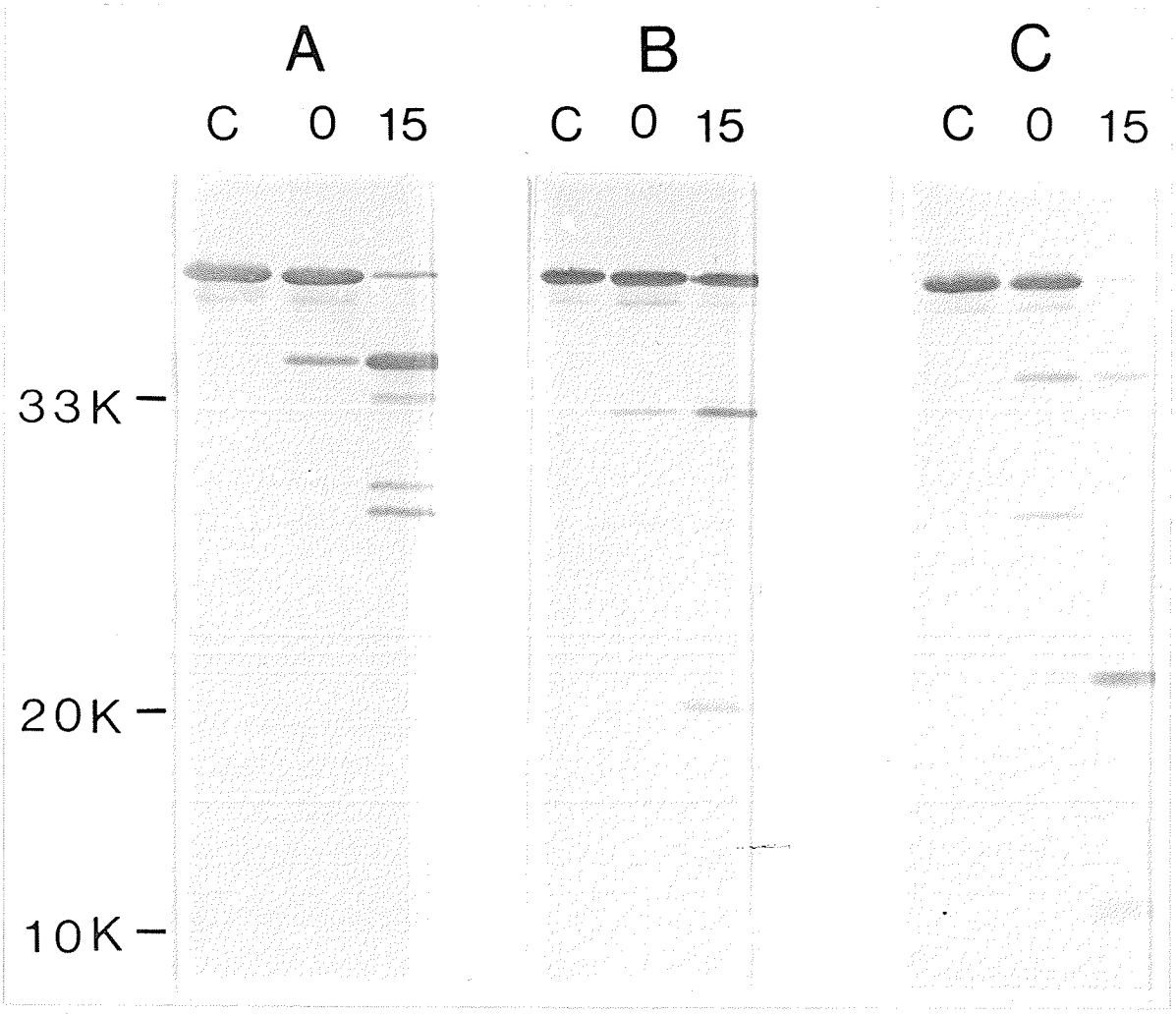


24 Kd with the corresponding smaller fragments. The 33 Kd fragment MW has only the two epitopes which bind the UM-3 and UM-4 MAbs, suggesting that a 2 Kd fragment has been lost which has the UM-1 epitope. The 25 Kd fragment (which is quickly destroyed) also has the UM-3 epitope but not the UM-4 epitope. The 24 Kd fragment persists and has only the UM-4 epitope. The trypsin apparently no longer has access to the MOMP, suggesting this 24 Kd fragment must be buried in the membrane.

A comparison of the fragmentation patterns seen with trypsin treated EBs from serovars L2, G and E is shown in Figure 25. In part A, serovar L2 was immunoblotted with the MAbs UM-1, UM-3 and UM-4 showing up the four major fragments from 35 Kd to 24 Kd. Part B of Figure 25 shows serovar G developed with MAb UM-3. There are two major fragments by 15 minutes, one at 33 Kd and another around 20 Kd. When the MAb UM-2 (subspecies recognizing serovars C, G and F) was used, the same pattern was observed. Of interest, the serovar G MOMP was more resistant to trypsinization than serovars L2 and E. In part C, serovar E is shown with three sets of reactive fragments at 35 Kd, 20 Kd and 10 Kd. The 20 Kd fragment is the most dominant but does not persist past 90 minutes. Again, with both serovars G and E, a sequential breakdown pattern is seen. A specific high molecular weight fragment is always seen before the lower molecular weight fragments appear.

At this time, we had been treating the EBs with trypsin and running the whole suspension on gels, making no attempt to separate soluble material. We therefore did not know if the trypsin was releasing portions of the MOMP which still retained their reactivity with MAbs. To look at this question, we treated whole EBs from serovar L2 with trypsin for up to 150 minutes and then spun the suspension in a microfuge for 15 minutes. Both the pellet and supernatant fractions were run and blotted with the three

FIGURE 25. Immunoblot of whole EBs (A: serovar L_2; B: serovar G; C: serovar E) treated with trypsin, separated on 12% PAG and developed with MOMP MAbs UM-1, UM-3 and UM-4 (A), UM-3 (B), and UM-2 (C). The time period for protease treatment is given in minutes across the top (C is an untreated control) and molecular weight estimates are listed on the left.



MAbs, UM-1, UM-3 and UM-4. The silver stained PAG is seen in Figure 26 and the immunoblot is seen in Figure 27. The silver stained gels shows only three bands in the supernatant fractions which are the trypsin molecule and its breakdown products. The immunoblot is most instructive as none of the MAbs bind to material found in the supernatant fraction. This suggests that while the trypsin makes cuts in the MOMP, the resulting fragments are still held to the EB or if fragments are released, they are immunologically non-reactive.

One way in which MOMP peptides could be held to the EB would be through disulfide bonding with other fragments or protein imbedded in the outer membrane. We repeated the same set of experiments except that whole EBs were treated with trypsin and the pellet resuspended in either plain buffer or buffer with 20 mM DTT. The EBs were incubated for 30 minutes and then spun down again and the pellet and supernatant fractions run on PAG for silver staining and immunoblotting. Again, no antigenic material which reacted with the MOMP MAbs was seen in the supernatant fractions. However, the 60 Kd, 57 Kd and 18 Kd antigens previously described as being released by DTT were seen in the supernatant fraction. These data would suggest that disulfide bonds are not responsible for holding the MOMP together after trypsin treatment and that the fragments must be imbedded in the membrane.

Essentially all of this information suggests that trypsin makes specific cuts in the MOMP causing it to open up to a certain extent for a second round of cuts to be made. This process is only visualized when the EBs are solubilized and run on a PAG which separates the peptide fragments. After trypsin treatment, the EB apparently remains intact as it can be spun out of suspension without any loss of outer membrane fragments. We therefore wanted to see if the EBs were still viable as measured by infec-

FIGURE 26. Silver stain of 15% PAG. Whole serovar L_2 EBs were treated for varying time periods with trypsin then spun in a microfuge to give pellet (P) and supernatant (S) fractions which were run on the PAG. The time periods in minutes are given across the top (C is an untreated control) with the molecular weight estimates listed on the left.

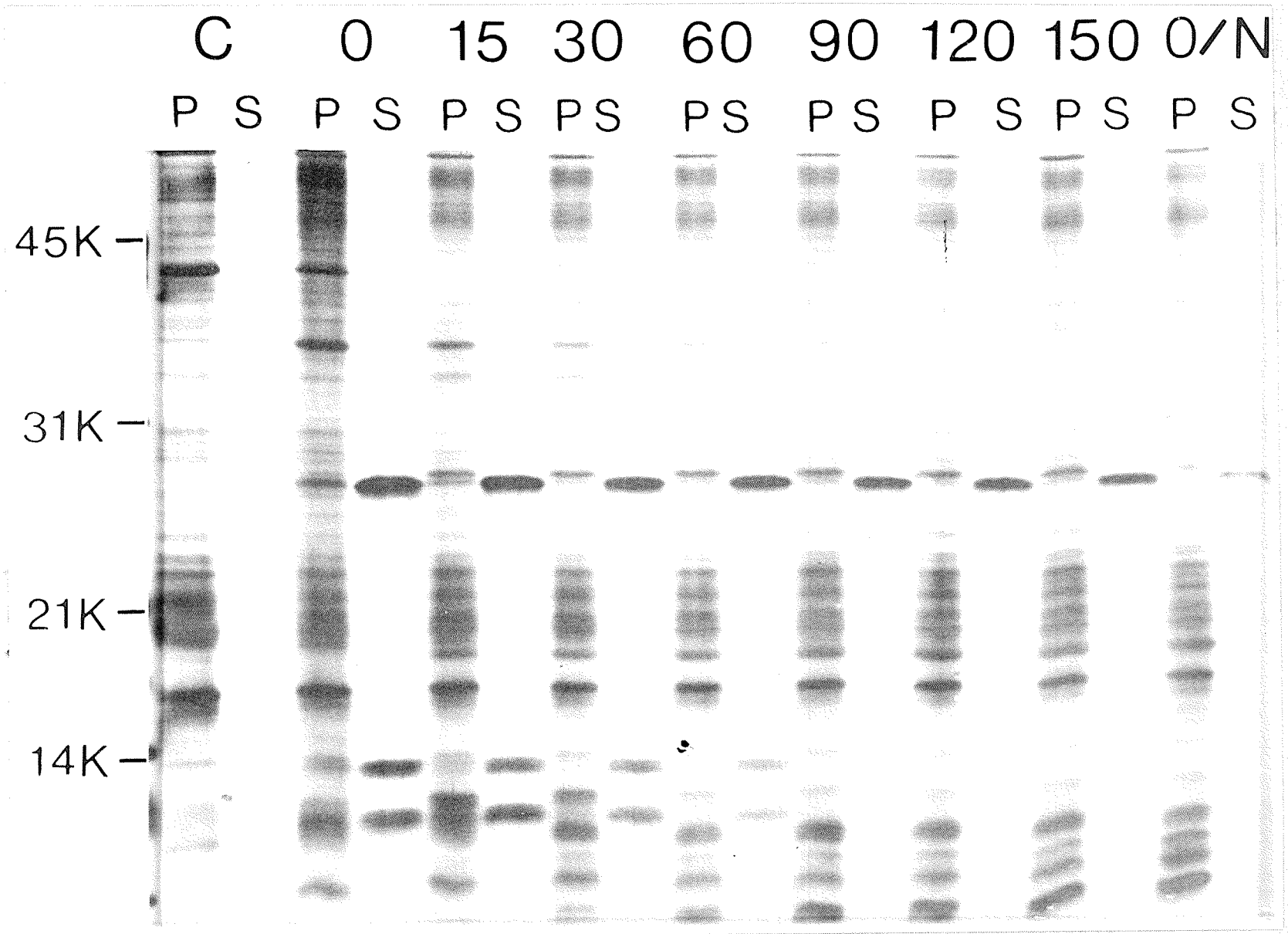
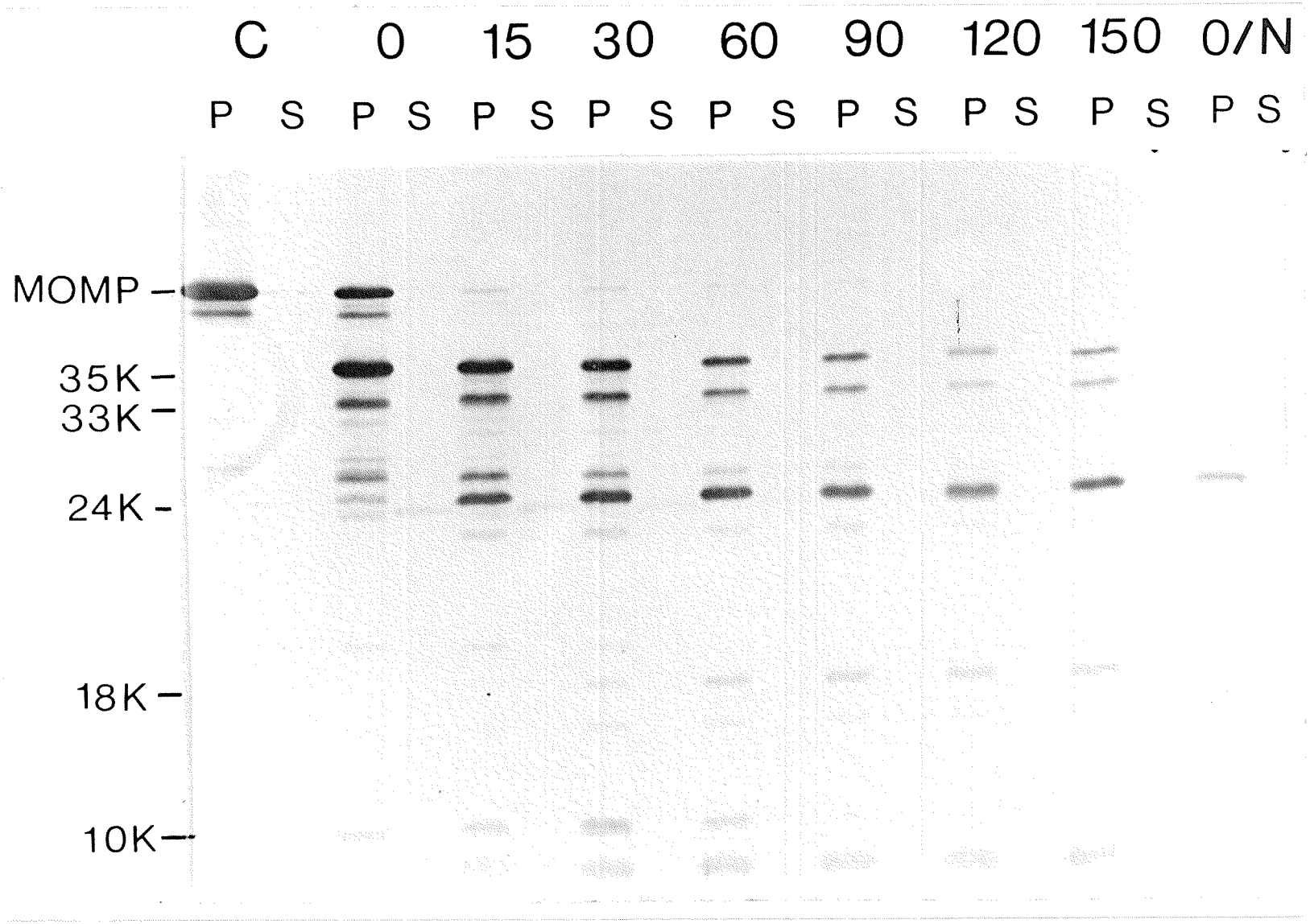


FIGURE 27. Immunoblot of the gel from Figure 26 developed with the MOMP MAbs UM-1, UM-3 and UM-4.



tivity. We treated whole EBs with three different amounts of trypsin for various time periods, inactivated the trypsin with PMSF and then used this suspension to infect HeLa cell monolayers. The level of infectivity is shown in Figure 28 while the cleavage of the MOMP can be followed in Figure 29. Essentially, the EBs were twice as infective at 15 minutes compared to untreated controls and then became less infective after two hours. We tried to correlate this increase in infectivity with the breakdown pattern of the MOMP, but no correlation was seen. The other non-MOMP MAbs were used and again, no increase or decrease in blotting patterns could be correlated with the initial increase then subsequent decrease in infectivity (data not shown).

11. Isolation of MOMP Peptides

The treatment of whole EBs with trypsin did not release detectable amounts of the MOMP and the EBs maintained their infectivity. This suggested that the outer membrane was remaining intact and functional. We therefore trypsinized whole EBs and then isolated the outer membrane complex by extraction with 2% sarkosyl which resulted in MOMP peptides free of contaminating cytoplasmic proteins. The peptides were cut only where there were surface exposed trypsin sites thereby identifying surface exposed regions of the MOMP. An example of the results obtained using serovar D are seen in the silver stain in Figure 30. The untreated outer membrane profile shows the MOMP and the 57 Kd antigen. The trypsinized outer membrane complex has six major bands. There is a 52 Kd peptide which is a result of trypsin cleavage of the 57 Kd antigen. It is immunologically active as can be seen in Figure 31. The dominant MOMP peptides are seen at 26 Kd, two around 20 Kd, 17 Kd and one at <10 Kd. When antisera

FIGURE 28. Graph showing the level of infectivity for serovar L_2 EBs treated with three concentrations of trypsin as compared to untreated EBs over a two hour time period.

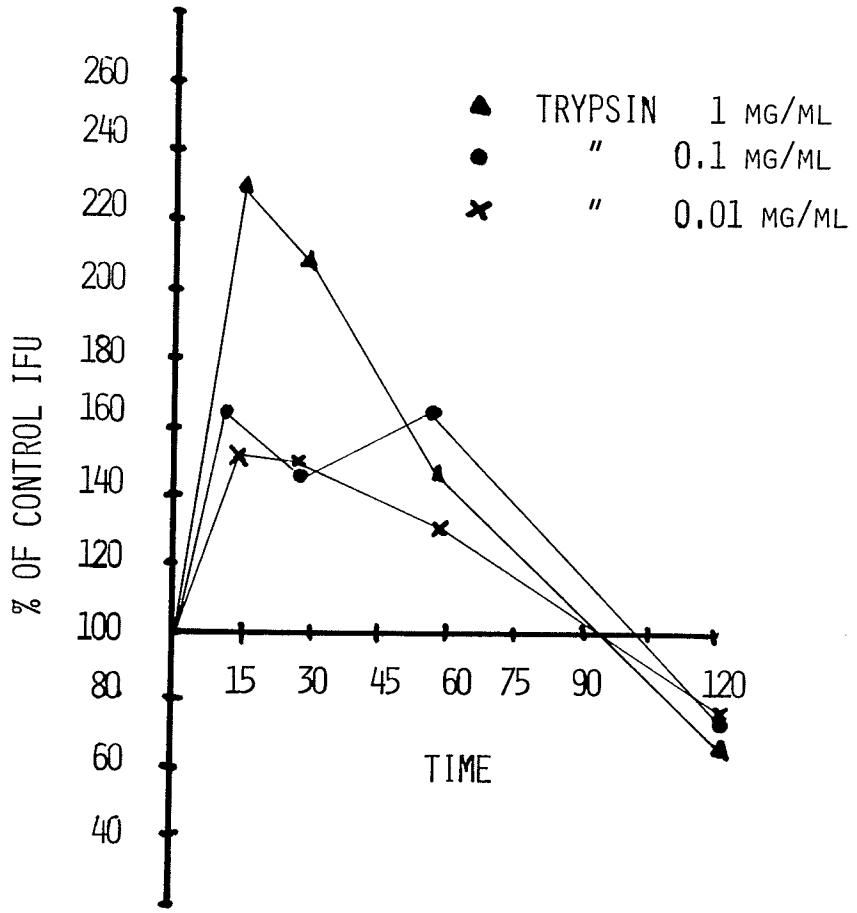


FIGURE 29. Immunoblots of whole serovar L_2 EBs treated with three concentrations of trypsin, separated on 12% PAG and developed with MOMP MAbs UM-1, UM-3 and UM-4. The time periods of digestion are listed with the trypsin concentrations being (a) untreated control, (b) 1 mg/ml, (c) 100 ug/ml, and (d) 10 ug/ml.

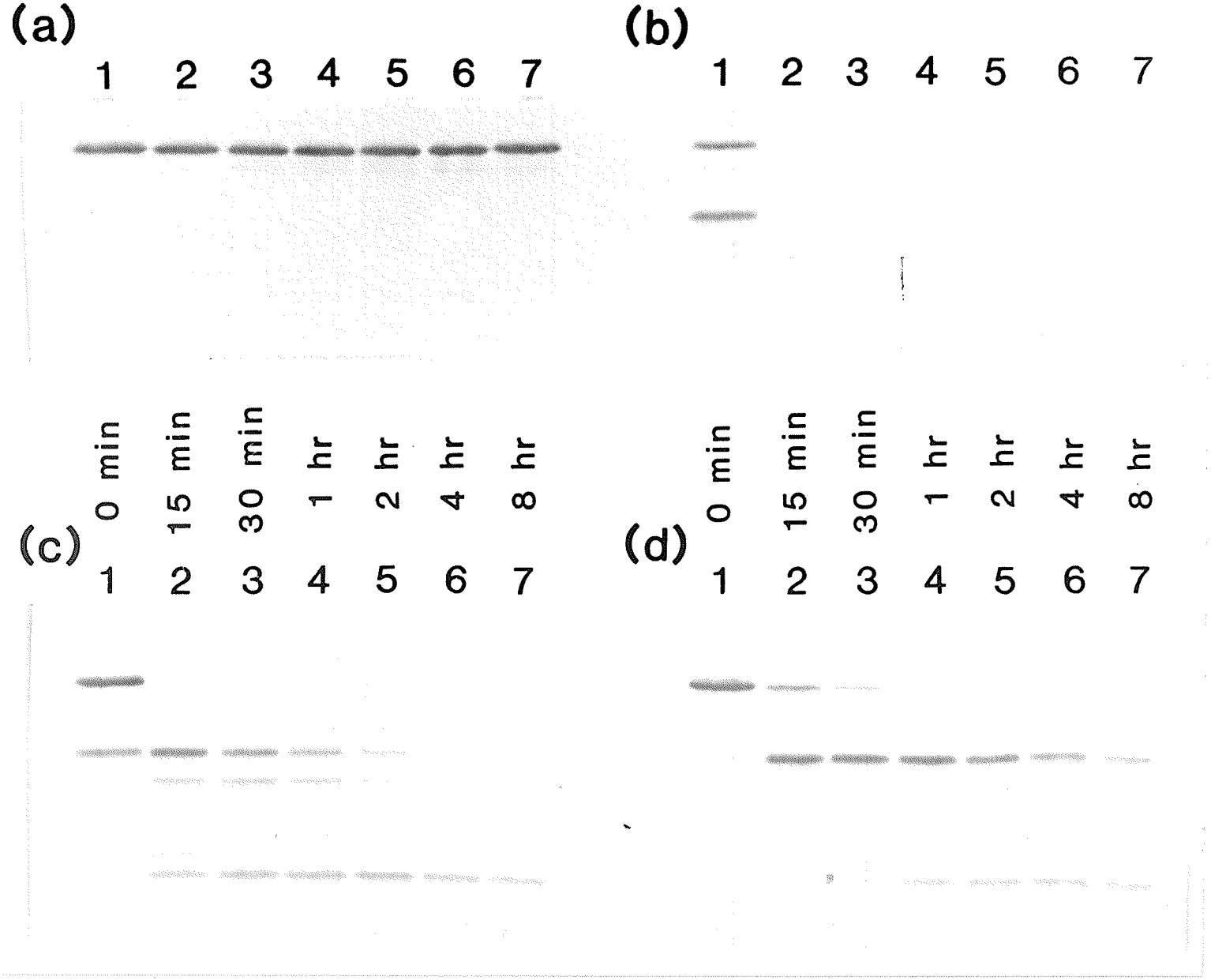


FIGURE 30. Silver stain of a 12% PAG. Lane A: Sarcosyl insoluble outer membrane complex from serovar D. Lane B: Sarcosyl insoluble outer membrane complex isolated after trypsin treatment of serovar D EBs. The MW of the proteins and peptides are listed on the left and right, respectively.

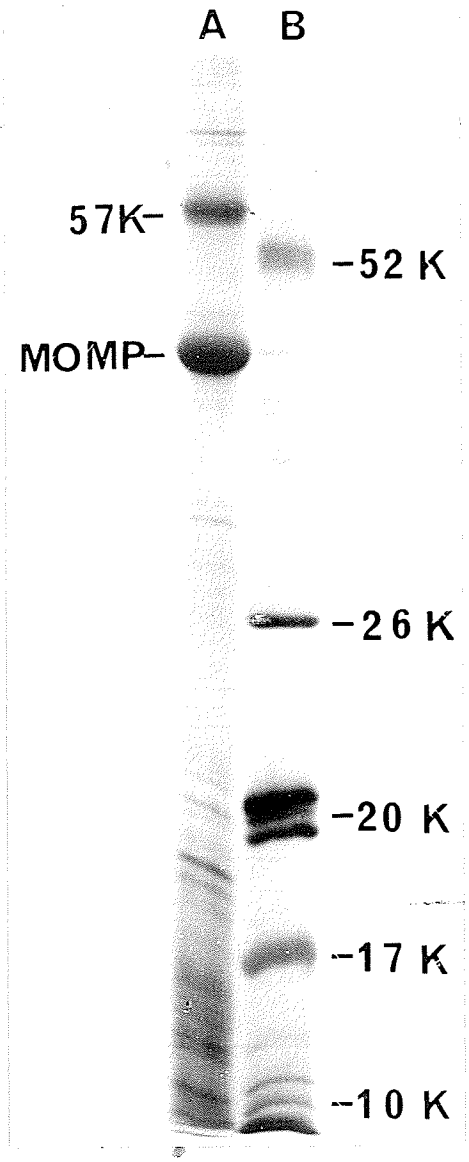
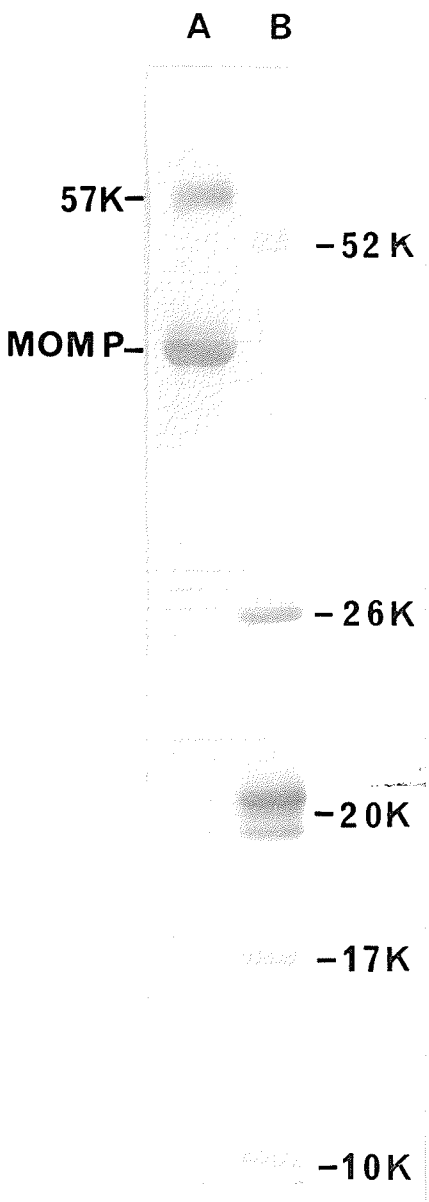


FIGURE 31. Immunoblot of a 12% PAG developed with sera from mice immunized with serovar D outer membrane complex. Lane A: Sarcosyl insoluble outer membrane complex from serovar D. Lane B: Sarcosyl insoluble outer membrane complex isolated after trypsin treatment of serovar D EBs. The MW of the proteins and peptides are listed on the left and right, respectively.



prepared against the outer membrane complex was used in an immunoblot, all of these peptides were reactive.

These data suggest that there are two highly susceptible areas on serovar D MOMP which are attacked by trypsin. One would be the site joining the 26 Kd and 17 Kd peptides which together add up to the 40-43 Kd MW for MOMP in general. There is a second site in the middle of the MOMP molecule resulting in a doublet with MW ranging in size from 19-22 Kd. There are also a number of other minor peptides seen indicating susceptible sites but access to them is more restricted.

Because the MOMP peptides were essentially free of other proteins, we undertook to separate them on PAG and transfer them to activated glass fibre for the purposes of amino acid sequencing. The peptides were also separated using an HPLC. Overall, three sequences were obtained. The HPLC isolated a low MW peptide from which we obtained the first 25 amino acid residues. When this sequence was compared to the published sequence of serovar L_2 MOMP, it began at amino acid number 310 (Figure 32) (Stephens et al, 1986). Portions of the 26 Kd and 17 Kd peptides were sequenced by first transferring them to glass fibre and then applying the protein-membrane complex to the protein sequencer. The doublet at 20 Kd could not be separated sufficiently to allow clear cut sequence data. The amino acid sequence from the 26 Kd and 17 Kd peptides is seen in Figure 32. The 17 Kd peptide is the amino terminus of the MOMP while the 26 Kd peptide lines up with the serovar L_2 sequence starting at amino acid number 147.

12. Protease Treatment of Solubilized MOMP

We set out to identify peptide fragments from serovar L2 MOMP which were reactive with MOMP MAbs. The MAbs included the one type specific and two subspecies specific MAbs against L2 MOMP produced in our laboratory and

FIGURE 32. Amino acid sequences for three serovar D MOMP peptides produced by trypsinization of whole EBs.

1. HPLC Purified Peptide

   310[a] Thr.Gly.Ala.Glu.Gly.Gln.Leu.Gly.Asp.Thr.Met.Gln.

   322 Ile.Val.Ser.Leu.Gln.Leu.Asn.Pro.Arg.Ser.Ala.Arg.Asp.

2. 17 Kd Peptide

   1 Leu.Pro.Val.Gly.Asn.Pro.Ala.Glu.Pro.His.Leu.Met.

   13 Ile.Asp.Gly.Ile.Leu.Pro.Glu.Gly.Phe.

3. 26 Kd Peptide

   147 Val.?[b].?.Ala.Ser.Val.Pro.Asn.Met.Ser.Phe.Asp.Glu.

   160 Ser.Val.Val.Glu.Lys.Tyr.Thr.Asp.Thr.Thr.Phe.

[a] Amino acid from serovar L2 MOMP (Stephens et al, 1986) to which these peptides correspond.

[b] Unknown amino acid residue.

one species specific MAb produced in James Mahoney's laboratory in Hamilton, Ontario. Our immunoblot using this MAb against 12 of the C.trachomatis serovars and the MoPn and CpMn strains is seen in Figure 33. There is no response to the MoPn or CpMn strains and a poor, but positive, response to the serovars A, D and E. The genus specific MAb against the MOMP was not used due to its weak response in the immunoblot and the restricted reaction by MIF.

The MOMP was prepared by first treating whole EBs with sarcosyl and then solubilizing the MOMP from the sarcosyl insoluble outer membrane complex using SDS. This solution also contained a minor amount of contaminating protein consisting mainly of the 57 Kd protein.

On initial test runs V8 protease and trypsin were used to cleave the MOMP. The trypsin was very active in a short period of time producing a large number of peptide fragments which were reactive with both the type specific and subspecies specific MAb (Figure 34). The peptide patterns are the same for the larger fragments as both epitopes are on the same fragment. Differences appear as the peptide fragments become smaller. The subspecies epitope appears to remain functional on a smaller fragment than that of the type specific eptiope. The silver stain of this gel shows a large number of peptides in the <10 Kd region which does not have alot of activity on the immunoblots. Because of the inability to identify a specific reactive fragment, we went back to V8 protease which is much less reactive against the MOMP.

Initially, the solubilized MOMP was reacted for up to nine hours with the V8 protease run on a PAG and then immunoblotted with a pool of the three serovar L2 MOMP MAbs. The blot is seen in Figure 35. There are essentially 10 highly reactive peptides with a M.W. range from 16 Kd up to a little less than 40 Kd. This appeared to be a much more manageable number of fragments to handle.

FIGURE 33. Immunoblot of 12 C.trachomatis serovars, the mouse pneumonitis and C.psittaci Mn strain separated on a 10% PAG and developed with a species specific MOMP MAb (J. Mahoney, Hamilton, Ontario).

FIGURE 34. Immunoblot of purified serovar L_2 MOMP treated with trypsin, separated on a 12% PAG and developed with MOMP MAbs UM-4 (A) and UM-3 (B). The time of protease treatment is listed in minutes across the top and the two molecular weight estimates are on the left.

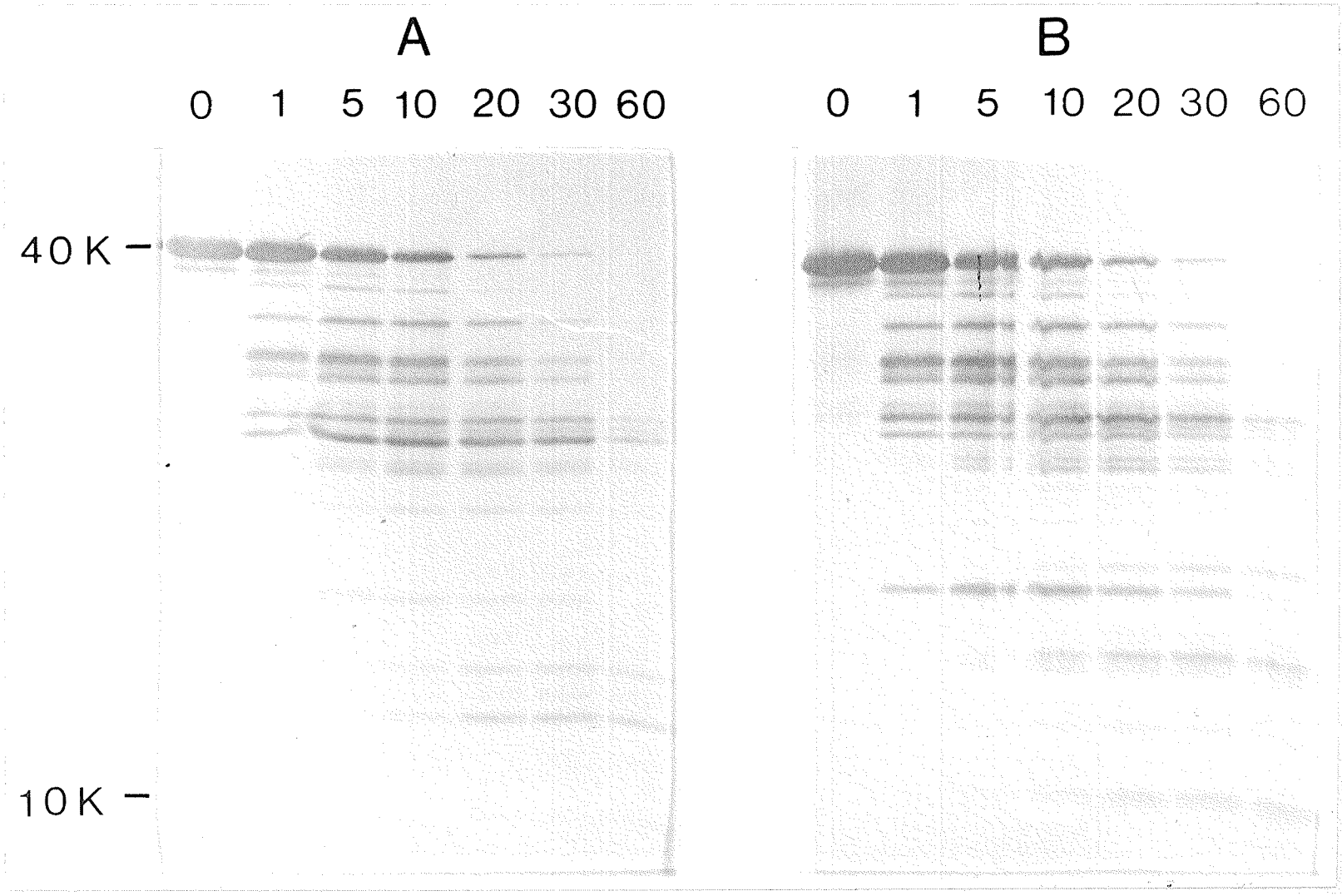
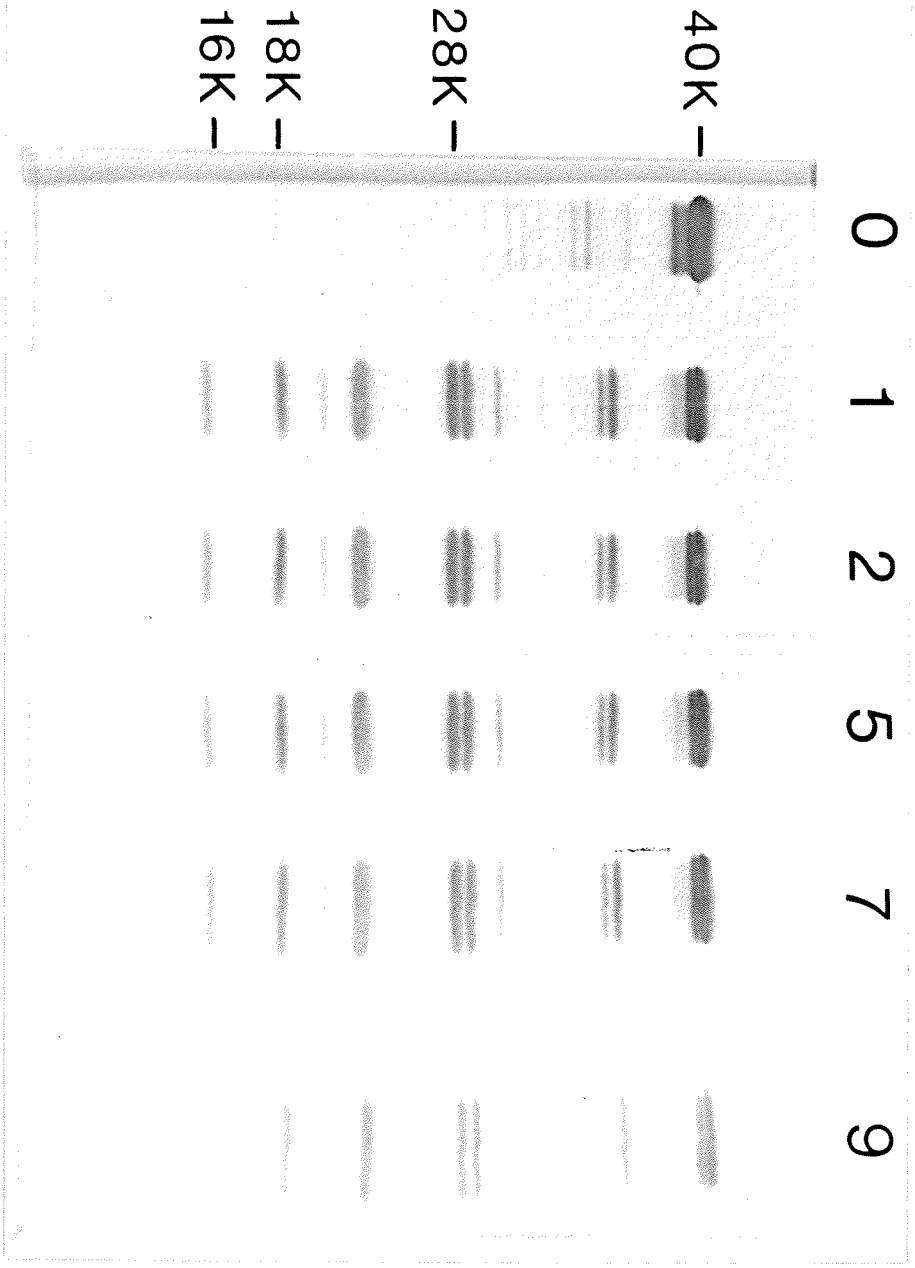


FIGURE 35. Immunoblot of purified serovar L_2 MOMP treated with V8 protease, separated on a 12% PAG and developed with MOMP MAbs UM-1, UM-3 and UM-4. The time periods of protease treatment are listed in hours across the top and the molecular weight estimates are given on the left.



The V8 protease digestion of MOMP was repeated using more enzyme to try and produce a more complete cleavage. When separated by PAG and immunoblotted, the blots were developed with the individual MAbs as seen in Figure 36. As well as the three MOMP MAb from our laboratory, the species specific MAb from Jim Mahoney was also used. Better cleavage of the MOMP was obtained as by 90 minutes, there is no intact 40 Kd MOMP remaining. The high M.W. fragments retain all the epitopes in a functional state. A difference in the blotting pattern can be seen around 28 Kd. The subspecies and species specific MAbs (Figure 36 B,C and D) recognize two fragments; the type specific MAb (Figure 36A) blots only one of them. This difference continues as the type specific epitope is found on fragments of 21 Kd, 16 Kd and 13 Kd while the other three epitopes are found on fragments of 22 Kd, 18 Kd, 14 Kd and 12 Kd. The species specific epitope is not found on the 14 Kd and 12 Kd fragments.

The smaller fragments do not bind the MAbs with the same intensity as the larger fragments. For example, if the 13 Kd fragment is a product of the cleavage of the 16 Kd fragment, it retains only about 25% of the ability to bind the MAb. The same can be said for the other small fragments. The 14 Kd and 12 Kd fragments which probably result from the cleavage of the 18 Kd fragment lose almost all of their ability to bind the MAb.

To try and better follow the digestion of the MOMP, we radiolabelled the solubilized MOMP with ^{125}I. Both labelled and unlabelled MOMP were treated with V8 protease, separated on PAG and developed on the immunoblot with MOMP MAb UM-3. The immunoblot was autoradiographed for 24 hours. On the immunoblot (Figure 37), the main fragments at 18 Kd and 14 Kd are seen to be the most intensely developed. The autoradiograph (Figure 38) shows a band at 18 Kd and two large bands at 14 Kd and 12 Kd. The intensity of the blotting is not in a direct relationship to the amount of material present

FIGURE 36. Immunoblots of purified serovar L_2 MOMP treated with V8 protease, separated on a 15% PAG and developed with MOMP MAbs UM-4 (A), UM-1 (B), UM-3 (C) and a species specific MOMP MAb (D). The protease treatment times are listed in minutes across the top with the molecular weight estimates given on both the right and left sides.

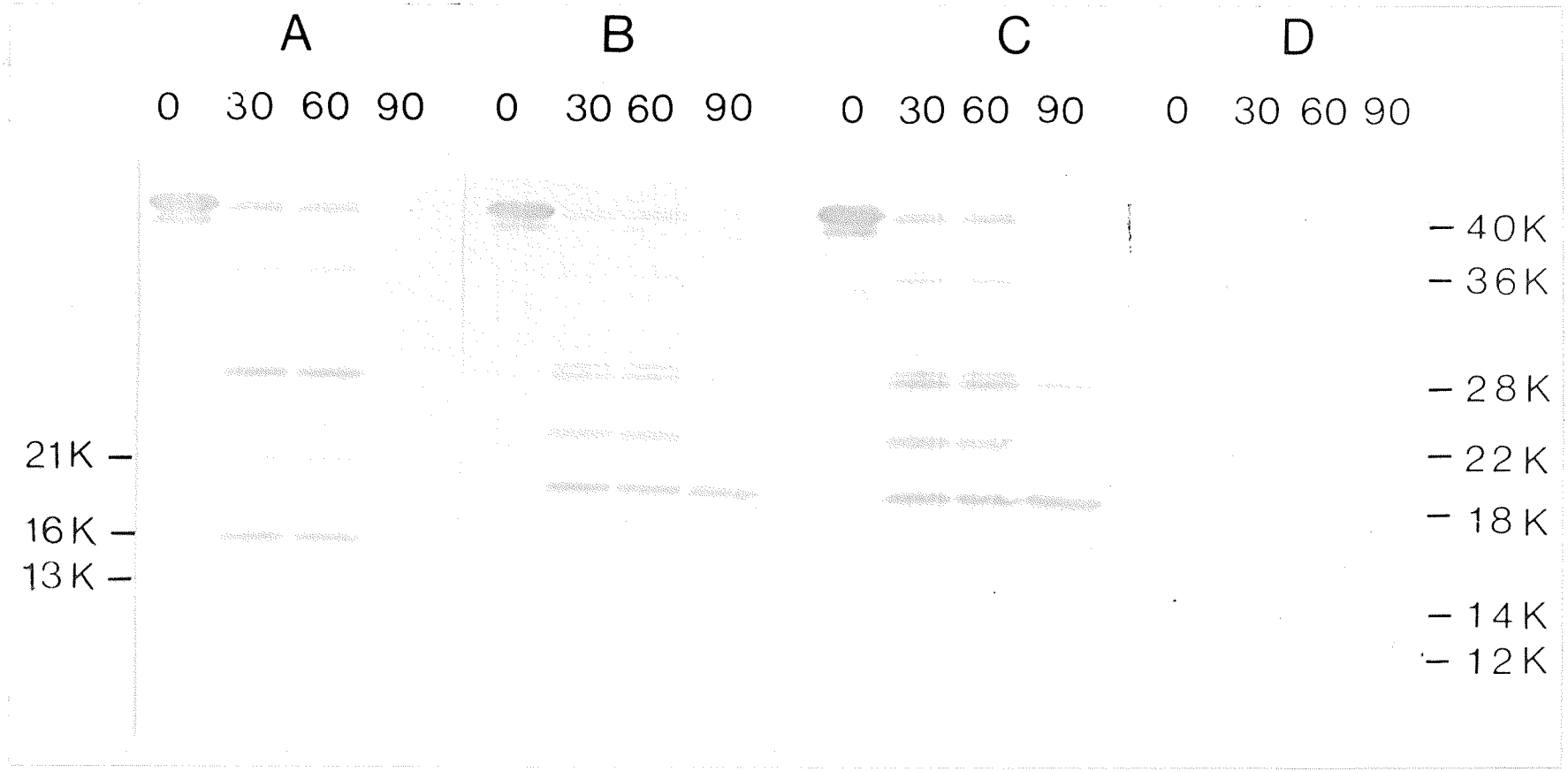


FIGURE 37. Immunoblot of purified serovar L_2 MOMP labelled (0, 30, 60, 120) and unlabelled (0', 30', 60', 120') with ^{125}I, treated with V8 protease, separated on a 15% PAG and developed with MOMP MAb UM-3. The time period of protease treatment is given in minutes across the top and the molecular weight estimates are given on the left.

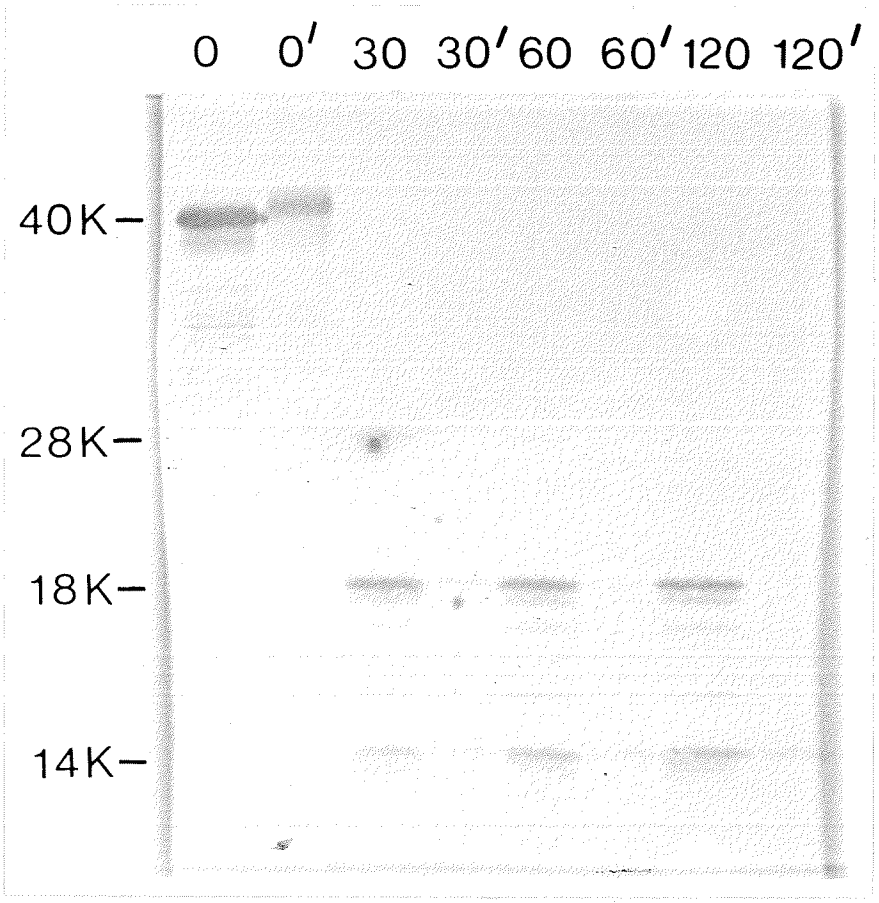
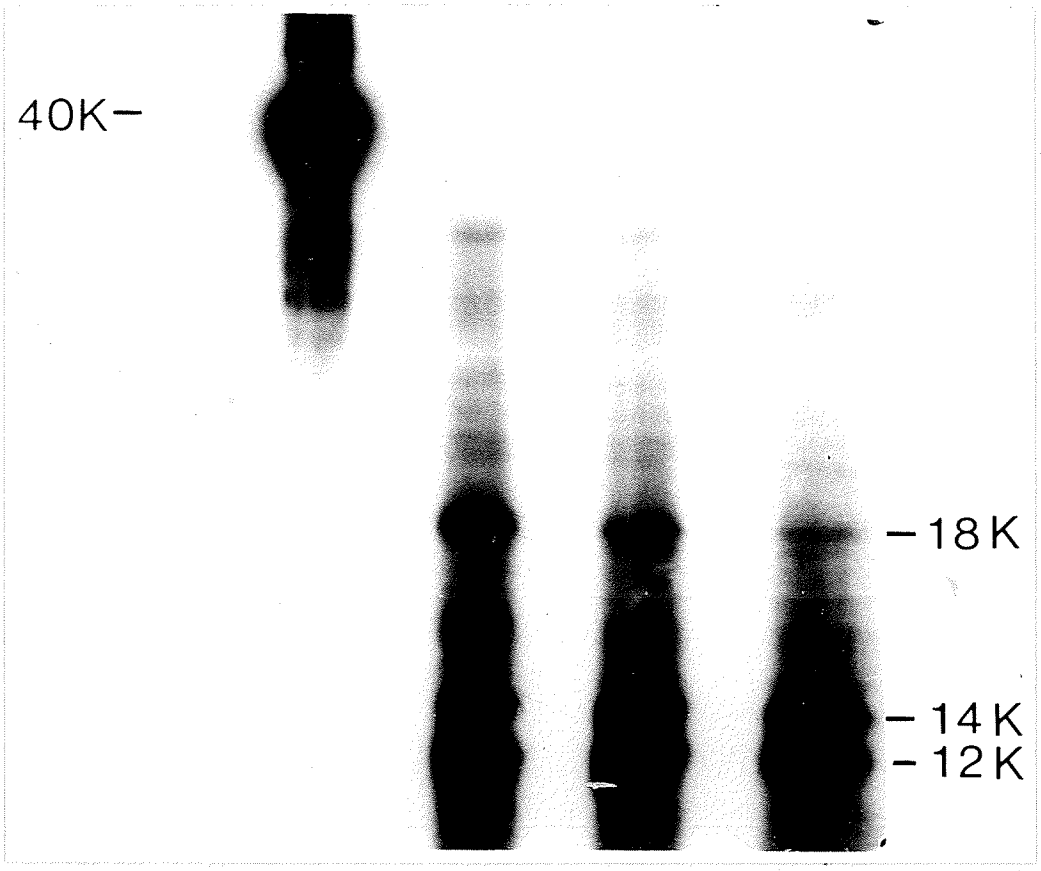


FIGURE 38. Autoradiograph of the immunoblot in Figure 37. The untreated MOMP is seen at 40 Kd (left side) while the molecular weight of the breakdown products are seen on the right side.



suggesting most of these fragments do not have reactive epitopes. Other fragments unrelated to the UM-3 fragment could also be mixed in with this group of peptides.

DISCUSSION

We undertook a detailed study of outer membrane antigens of C.trachomatis. Multiple approaches were used in the pursuit of this goal. We characterized antigens by immunoaccessibility, accessibility to protease digestion, mild extraction with dithiothreitol and insolubility in sarcosyl and identified antigen specificity by Western blotting. We were particularly interested in outer membrane protein antigens which are shared among members of the genus Chlamydiae and which induce neutralizing antibody. The overall results of these studies are shown in Table 10. Considered in aggregate, the data suggest that at least eight protein antigens are found in the outer membrane of C.trachomatis. These antigens vary markedly in accessibility as part of the intact EB to antibody and in antigen specificity. Two genus specific antigens were immunoaccessible and had neutralizing epitopes. These are the MOMP and 75 Kd antigens.

When we began our studies on the antigens of C.trachomatis, silver stained gels of solubilized EBs showed proteins which appeared common between serovars and others which were restricted to a few serovars. The only components known to be common between the two chlamydial species were the MOMP and LPS. Because the two species do share a similar unique life cycle, we reasoned there may be common proteins between them which have been conserved as they perform essential functions.

1. Serological Response to Chlamydial Antigens

Immunoblotting of serovars L2 and D with sera from women with natural chlamydial infections was used to identify antigens which might be important in the infectious process. We used rabbit anti-human antibodies tagged with horseradish peroxidase and diamino benzidine as the colour reactant to develop our immunoblots. This allowed the clear delineation of any anti-

TABLE 10. Outer membrane proteins of C.trachomatis.

Molecular Mass ($x10^3$)	Antigenic Specificity	Immunoaccessibility[a]	Protease Accessibility	Dithiothreitol extractability	Sarcosyl Insolubility
75	Genus	+	+	+	-
70	Genus	-	+	-	-
60	Species	-	+	+	-
57	Species	-	+	+	+
40 (MOMP)	Genus				
	Species	+	+	-	+
	Subspecies				
	Type				
32	Species	-	+	-	-
29	Species	+	+	-	-
17	Genus	ND	+	+	-

[a] Ability of antibody to the antigen to bind to an intact EB.

ND = Not Done.

body response to a specific antigen without the more dominant responses blocking out the lesser ones as happens when ^{125}I-protein A autoradiography is used.

While in some cases the main response was directed against the MOMP, many other antigens were recognized by the serum antibodies and, in some cases, were more prominent than those towards the MOMP. Besides the MOMP and LPS, we identified reactions with antigens of 100 Kd, 90 Kd, 75 Kd, 60 Kd, 57 Kd, 45 Kd, 32 Kd and 29 Kd. Whereas the response to the MOMP epitopes was often type specific, in many responses to the other antigens, there appeared to be a uniform species specific reaction suggesting species specific epitopes on these antigens. One interesting aspect of this study was that the 57 Kd antigen was immunodominant in a majority of women with tubal infertility as compared to seropositive pregnant women (Brunham et al, 1985). This result suggests that while the MOMP is the prominent outer membrane antigen, other antigens may have more important roles to play in the pathogenesis of chlamydial infection.

Newhall et al (1982) were the first to immunoblot chlamydial antigens. They used sera from women with chlamydial genital infections and showed a response to the MOMP and antigens of MW 62 Kd, 60 Kd, 29 Kd and, occasionally, a 15 Kd antigen. Because controls reacted with the MOMP and 29 Kd antigens, the authors felt that the 60 Kd doublet might be an important component in the infectious process. This doublet is most likely what we recognize as the 57 Kd antigen. Inman et al (1987) also reported a strong response with a 59 Kd antigen from sera of Reiter's syndrome patients in relation to the MOMP. Brunham et al (1987) have shown that a lack of IgG to antigens of 75 Kd, 60 Kd and 57 Kd may increase the chance of ascending pelvic infection in women, while the presence of antibodies to a 100 Kd, 32 Kd and 29 Kd antigens may prevent upper genital tract infec-

tion. The presence or absence of antibodies to the MOMP did not correlate with the risk of ascending upper genital tract infection. However, since homologous strains were not used, the role of type-specific antibodies was not addressed. In patients with LGV disease, all sera had significant amounts of antibody to the MOMP with varying responses to 17 Kd, 29 Kd, 60 Kd, 65 Kd, 128 Kd and 150 Kd antigens (Ward et al, 1986). The trachoma sera evaluated in the same study tended to be of lower titre and had poor responses to the MOMP but there were good responses to antigens around 60 Kd.

Immunoblotting of sera from monkeys with experimental conjunctivitis showed tear IgA responses to the MOMP and antigens of 60 Kd and 68 Kd (Caldwell et al, 1987). The serum IgG response was similar. Batteiger and Rank (1987), using a C.psittaci strain, showed a humoral response to MOMP and 61 Kd and 84 Kd during an experimental guinea pig genital infection. Although the 84 Kd antigen is hard to align with other C.trachomatis antigens, the 61 Kd antigen appears to be similar to with the 57 Kd, 59 Kd and 60 Kd doublet described by ourselves and other authors showing that it may be a genus specific antigen and important in the infectious process.

After establishing the antigens to which we wanted MAbs, an immunization schedule was set up to duplicate the humoral response seen in humans. Initial immunizations, which relied on intravenous injections only, tended to have a response directed against the MOMP and LPS. In order for the lesser antigens to be recognized, intraperitoneal and subcutaneous injections with antigen in Freund's adjuvant were used. This produced a strong response to a wide variety of antigens and exceptional responses to the MOMP. In three different sets of mice, the immune titres, as assayed by MIF, were 1:12,500, 1:25,000 and >1:25,000 for serovars L2, J and G, respectively. Immunoblotting of this sera at 1:500 showed strong genus

specific responses to the MOMP and reactions with at least 15 other antigens, both species and genus specific.

At this point, one would ask why only certain antigens elicit an immune response? The silver stained gels show a large number of proteins to which no detectable immune response is seen. This would suggest then that the antigens which induce a response are probably surface exposed or part of the outer membrane such that they would come into contact with the immune system in a more immunogenic form. We used intact untreated whole EBs for immunization as this would mimic a natural infection to some extent and any MAbs isolated would be against naturally occurring epitopes, whether they were surface exposed or seen after immunological processing by the mice. No matter what method we used to identify them, the MAbs were recognizing naturally occurring epitopes.

2. Characterization of Monoclonal Antibodies Raised to Eight Chlamydial Antigens

We carried out three fusions using two different methods to screen the hybridoma cells. The ELISA assay employed whole EBs and so would detect antibodies directed against surface exposed epitopes. The second assay used NCM strips with resolved chlamydial proteins to identify the antibodies reacting with the lesser antigens. Of all the hybridoma lines we isolated, we focused our attention on 13 clones. Six produced antibodies specific for the MOMP, one was specific for the LPS and six others were directed against antigens of MW 29 Kd, 32 Kd, 57 Kd, 60 Kd, 70 Kd and 75 Kd.

The MOMP MAbs had type, subspecies and genus specificity by immunoblot. The type specific MOMP MAb was directed against the L2 MOMP. Such antibodies have been described by other authors but it is unknown how many

type specific regions each MOMP has, so this MAb may have unique characteristics. We did not obtain any type specific MAbs to the J or G MOMPs which is a case of not using the right screening system. The two subspecies MOMP MAbs which have been previously described (Stephens et al, 1982) recognized serovars L2, D, E, B and serovars C, J, I, A and H. These two epitopes represent the two serological groups of the B and C complex as defined by Wang and Grayston (1977), suggesting this must be a very immunodominant epitope. The two other subspecies MAbs recognized epitopes on serovars G, C, F and serovars L2, G, E and MoPn. Neither has been previously described. The G, C, and F relationship is not seen with polyclonal sera (Wang and Grayston, 1977), suggesting that this epitope, while surface exposed, is not immunodominant and is drowned out by more prominent epitopes. The other MOMP MAb is also interesting as it crosses the wide genetic gap between the human trachoma serovars and the mouse pneumonitis strain. Stephens and Kuo (1984) have previously reported that a species specific MAb also recognized the MoPn strain, so our data would indicate there is another common epitope. The same MAb also links the L2 and G serovars which were previously thought to be only distantly related. The final MOMP MAb isolated was genus specific on immunoblot but showed a restricted subspecies pattern by MIF assay. The genus specific reaction of the polyclonal sera shows that there are genus specific epitopes on the MOMP. Whether there are more than one per molecule is not known. The genus specific epitope our MAb recognizes may not always be surface exposed in all the serovars on MIF, but would be immunologically available after solubilization and Western blotting.

All of the MOMP MAbs were strongly positive in the ELISA assay and the MIF assay results essentially followed those seen by immunoblot except for the genus specific MAb. These results show that the epitopes on the MOMP

are surface exposed and may be linear epitopes as opposed to conformational epitopes. These antibodies were tested in a neutralization of infection assay. Only the subspecies MAbs against serovars L2, D, E, MoPn (UM-1) and G, C, F (UM-2) were effective. UM-1 was able to neutralize serovar L2 with and without complement but no other serovars. UM-2 showed only complement dependent neutralization with serovars F and G. All types of MOMP MAbs appear effective in neutralizing infection. Peeling et al (1984) used a species specific MAb, Fedorko et al (1987) used a subspecies specific MAb, while Lucero and Kuo (1985) showed that both type and subspecies specific MAbs to MOMP are effective for neutralization. Both Caldwell and Perry (1982), and Peeling et al (1984) demonstrated that the MOMP antibodies do not prevent attachment or uptake into the cell, so their effect takes place after internalization. Caldwell and Perry (1982) suggested that cross-linking of the outer membrane may prevent reorganization from the EB to the RB.

To date, neutralization with MOMP antibodies is only an in vitro situation. A recent paper by Taylor et al (1987) shows that an immunization schedule which gives high titres of anti-chlamydial antibody (assumed to be anti-MOMP antibody) will not prevent subsequent reinfection. One problem that I visualize with a MOMP vaccine is that penetration of the cell by the EB is not prevented. The EB could residue intracellularly until the neutralizing antibodies dissociate and then initiate infection. Zhang et al (1987) also suggests that species specific MAbs are ineffective in neutralizing infection; this is unfortunate as these epitopes would theoretically be the best vaccine candidates. This means a MOMP vaccine might have to be made up of the neutralizing epitopes from each serovar. It would appear much more logical to prevent attachment and uptake.

The LPS MAb we concentrated our efforts on was genus specific. With the full characterization of the LPS molecule carried out by other authors (Brade et al, 1985; Nurminen et al, 1985) and our data that the LPS MAbs did not have neutralization ability, we did not carry out any further analysis of the LPS MAbs.

The object of this thesis was to look for other antigens which induce an immune response. Using the immunoblotting assay of hybridoma supernatants, we were able to isolate MAbs to antigens of MW 29 Kd, 32 Kd, 57 Kd, 60 Kd, 70 Kd and 75 Kd. We used these MAbs to isolate two of the antigens in pure form and to characterize the antigens to some extent.

The two MAbs, UM-8 and UM-9, recognize antigens of 29 Kd and 32 Kd, respectively. These two antigens, other than LPS, are the only two which are consistently seen with immune sera in the MW range <40 Kd. Both antigens were common to all C.trachomatis serovars with no cross-reacting antigen seen with the MoPn strain or the C.psittaci strain using either the MAbs or the polyclonal sera against whole EBs. We would probably need good monospecific polyclonal sera which we were unable to obtain. The 29 Kd antigen had a constant MW for all serovars while the 32 Kd antigen was seen for serovar L2 only and had a slightly lesser MW for all other serovars. The 29 Kd antigen is most interesting in that it appears to be part of a doublet. This was seen using the polyclonal sera with the serovars A, B and C. They have both a 29 Kd and a 28 Kd antigen, while the other serovars have just a single band at 29 Kd on immunoblots. Our 29 Kd MAb is directed against an epitope on the antigen with the higher MW.

Our characterization of the 29 Kd and 32 Kd antigens showed them to be found, for the most part, in the sarcosyl soluble fraction from whole EBs, suggesting they were not part of the outer membrane. They were, however,

susceptible to attack by proteases which may mean parts of these antigens are surface exposed. The 28 Kd antigen did not appear to be cleaved by the proteases we used. Radiolabelling studies were not conclusive as to whether or not the 29 Kd and 32 Kd antigens were specifically being labelled. Both MAbs to these antigens were negative when used in the MIF assay, however, the 29 Kd MAb was positive in the ELISA assay while the 32 Kd MAb remained negative. Our conclusion from this information was that the 29 Kd antigen is surface exposed while the 32 Kd antigen is not surface exposed unless proteolytic agents are used to open up the outer membrane.

Two recent papers have described antigens of 31-32 Kd and 18 Kd which may be involved in the attachment of the EB to the eucaryotic cell. The 18 Kd protein was common to all chlamydiae tested but the 31-32 Kd protein in L2 migrated at molecular weight of 27 Kd in serovars D, G and H, and 23 Kd in serovar B. Only the 18 Kd antigen was common to C.psittaci strains. Radiolabelling was inconclusive as to whether these antigens were surface exposed and immune sera failed to react with either of these antigens on Western blot (Hackstadt, 1986a). Wenman and Meuser (1986) had immune sera which did react with these two antigens and found that monospecific sera to each of the antigens was able to neutralize infection.

It is hard to make a comparison between our 29 Kd and 32 Kd antigens and the 31 Kd antigens of Wenman and Meuser (1986) and Hackstadt (1986). We do not see the wide range in MW that Hackstadt (1986) describes while Wenman and Meuser (1986) suggest the antigen is part of the outer membrane, although the detergent solubilization data suggests otherwise. The 17 Kd antigen doublet we identified from the DTT treatment of whole EBs may be similar to the 18 Kd antigen, however, our data showed some MW variability within the genus while the other authors show a common MW for this pro-

tein. Our immune sera from both humans and animals never recognized the 17-18 Kd antigen.

The MAb, UM-10, binds to an antigen of 57 Kd. This antigen was found to be species specific, although the epitope which reacts with the MAb was common only to the C.trachomatis serovars and not with the MoPn strain. Monospecific polyclonal sera raised to the 57 Kd antigen purified using affinity columns did react with the MoPn strain but not C.psittaci. The 57 Kd antigen appears to be part of the outer membrane complex. When whole EBs were solubilized with sarcosyl followed by SDS, the 57 Kd antigen remained essentially insoluble unless reducing agents were added. There was also a small amount of the MOMP associated with this insoluble material. Extrinsic radio-iodination studies show the 57 Kd antigen to be specifically labelled and proteases added externally to whole EBs are able to cleave this antigen, resulting in a 50 Kd peptide with a functional epitope.

One of the great disappointments was that MAb and the monospecific polyclonal sera to the 57 Kd antigen were negative in both the ELISA and MIF assay. The sera were also unable to neutralize in vitro infection. In a way, it is interesting that this surface exposed outer membrane protein should be so elusive. It may be sitting just below the surface of the membrane or it may be sterically hindered by the MOMP and LPS from interacting with antibodies. There is easy access to the external environment as the 57 Kd antigen is released from the membrane when DTT is added to whole EBs. By comparison, there is little or no release of the MOMP.

Another interesting aspect of this antigen is the humoral immune response it raises in women who are infertile due to severe chlamydial infection. Whether it is a cause of the inflammatory response or just an indicator of the infection is not known. When the DTT solubilized antigens

were run on PAG and silver stained, there were a considerable number of antigens present but it was only the 57 Kd antigen and a 17 Kd antigen which induced a humoral response in mice. These data would suggest these two antigens are particularly immunogenic.

Newhall (1986, 1987) has carried out extensive studies on the 60 Kd antigens of chlamydia. He refers to them as the 60 Kd doublet. Our 57 Kd antigen is most likely the lower MW antigen of the doublet. Newhall (1987) has shown that the 60 Kd antigen is a cysteine containing protein. As the transformation from RB to EB progresses, there is increased cross-linking with the MOMP and a 12.5 Kd protein which help to make up the outer membrane complex. The 60 Kd protein was found to be species specific although there were differences in net charge between the LGV strains and the other serovars. Peptide maps show considerable differences between the two groups while the C.psittaci strain appears to have peptides in common with the C.trachomatis serovars but not the LGV strains. However, there was no antigenic relatedness between C.psittaci and C.trachomatis with only partial reactivity between the LGV strain and the other serovars using seven 60 Kd MAbs (Newhall, 1986). There was no mention as to whether these MAbs were reactive in an ELISA or MIF assay.

The MAb UM-11 recognizes a 60 Kd antigen which might be the upper molecule of Newhall's 60 Kd doublet. However, this antigen differs considerably from the 57 Kd antigen. It is easily solubilized in sarcosyl and does not appear to be cross linked through disulfide bonds with any other proteins. The MAb reacts with epitopes common to all C.trachomatis serovars and the MoPn strains but not C.psittaci. Both the MIF and ELISA assays are negative when using the MAb, suggesting the epitope is not surface exposed; however, the 60 Kd antigen appears to be cleaved by proteases

when added to whole intact EBs. This antigen is frequently recognized during natural infections but there was no correlation of the humoral response to this antigen and the severity of infection.

The last two MAbs for discussion are UM-12 and UM-13, which recognize antigens of MW 70 Kd and 75 Kd, respectively. These antigens are both genus specific. In both cases, there is a slight increase in MW when seen with the C.psittaci strain as compared to the C.trachomatis serovars. They are different in that there is no reactive epitope for the UM-12 MAb with the MoPn strain. One suggestion to explain the similar increase in MW would be that the 70 Kd antigen is an incompletely solubilized 75 Kd antigen which moves through the gel at a faster rate. However, both MAbs do not react with the other so the hypothetical conformation change must destroy one epitope and create a new one which seems unlikely. Also, the polyclonal sera to the 75 Kd antigen does not recognize the 70 Kd antigen.

Both antigens are in the sarcosyl soluble fraction of whole EBs. The ELISA and MIF assays were negative with the MAbs but when the monospecific polyclonal antisera to the 75 Kd antigen was used, the ELISA was positive but the MIF assay remained negative. This would suggest part of the 75 Kd antigen is available to the surface. The same serum was able to neutralize infection in vitro compared to normal mouse serum. The mechanism of neutralization is unknown.

The MOMP genus specific epitope is found on a molecule which has a tremendous antigenic variability between the species which does not appear to be the case with the 75 Kd antigen. Whether the 75 Kd antigen has been retained by the two species for functional or only structural purposes is not known but the neutralization data suggests it may play a role in the infectious process.

3. Protease Treatment of Whole EBs, Outer Membrane Complex and Purified MOMP

One of the traditional methods used to compare related proteins is peptide mapping. The proteins are cleaved chemically or enzymatically and the resulting fragments are separated in one or two dimensions. The resulting patterns are then compared looking for common fragments. With the advent of MAbs, this procedure can be taken one step further as the peptide fragments can be transferred to NCM and probed with the various MAbs which are reactive with the intact molecule. This is known as epitope mapping as it allows one to locate the region of the molecule which the MAb is recognizing and perhaps assign some sort of function.

Caldwell and Judd (1982) used V8 protease peptide maps of intrinsically and extrinsically labelled MOMP from five serovars to show that there were a number of common regions with differences showing up mainly in the surface exposed regions. HPLC separation of fragments following chymotrypsin treatment of serovar G MOMP gave an elution profile with almost 30 peaks with six being identified as surface exposed (Judd and Caldwell, 1985). No attempt in either report was undertaken to react the peptides with polyclonal sera or MAbs. These authors used purified MOMP and did not look at enzymatic digestion of the MOMP in intact EBs. Hackstadt and Caldwell (1985) looked at single time period digestion of whole EBs with a number of enzymes followed by Western blotting and development with anti-MOMP sera. A number of reactive peptides ranging from 8-30 Kd were seen on the immunoblot using trypsin. Of the smaller peptides, less than 15 Kd, only an 8 Kd peptide was reactive with the polyclonal sera suggesting destruction of many of the epitopes by the trypsin digestion. The most interesting aspect of this paper was that the infectivity of the EBs was not

affected. With our panel of MOMP MAbs, we undertook the epitope mapping of the MOMP from serovar L2. This included both soluble MOMP and MOMP as part of the intact EB outer membrane.

We carried out initial digestions on both whole EBs and purified MOMP using the enzymes V8 protease, trypsin and chymotrypsin. The V8 protease was ineffective on the whole EB as far as MOMP digestion was concerned. With all of the serovars tested, there appeared to be only one surface exposed V8 protease site. Serovar L2 was an exception with at least two or three sites, while the MOMPs from serovars B, C and D did not appear to be susceptible to V8 protease attack. Trypsin and chymotrypsin were very effective on the whole EBs with good activity against all of the serovars with no exceptions. This proved to be a problem with purified MOMP as too many fragments were produced to sort out the breakdown pattern.

V8 protease was used with purified MOMP as it gave a limited number of peptides while trypsin was used with intact EBs to make peptide and epitope maps of MOMP.

The V8 protease peptide maps were developed with the MAbs reactive with serovar L2 MOMP. Included was a species specific MAb obtained from Dr. J. Mahoney, McMaster University. The digestion pattern showed that there was a stepwise breakdown of the MOMP suggesting it was folded on itself such that some sites were initially hidden until partial cleavage had taken place. Disulfide bonds would still be intact as the MOMP was solubilized with SDS only before the digestion. The type, subspecies and species specific epitopes were found on the same fragments at higher MW. It was only when looking at smaller peptides of about 20 Kd that the epitopes were found on different fragments. The two subspecies and single species specific MAbs all bound to the same fragments down to about 10 Kd at which time there was a loss of reactivity with the peptides. We also

radiolabelled the purified MOMP to better follow the peptide fragments. The autoradiograph showed a large number of peptides <10 Kd which when immunoblotted, were essentially negative. This is similar to the result Hackstadt and Caldwell (1985) found where the trypsin fragments of <8 Kd did not react with the polyclonal sera. The data would suggest there is a certain amount of a conformational component to the MOMP epitopes as linear epitopes should remain functional on small peptides. The smallest peptide which had functional epitopes for the two subspecies and single species specific MAbs was about 12 Kd. The epitopes may be clustered on one part of the peptide which is surface exposed when found as part of the outer membrane.

The more interesting aspect of the protease digestion of MOMP was seen with the treatment of the whole EB. We focused on serovar L2 but made some comparisons with serovars that were reactive with at least two MOMP MAbs. When looking at serovars C, E and G, any peptide fragments produced always had the epitopes for both of the subspecies epitopes. However, the peptide maps were by no means similar suggesting that the trypsin sites were found on different areas of the surface exposed portions of the MOMP.

We looked at the timed digestion of the whole serovar L2 EBs with trypsin following the breakdown of the MOMP with the type and subspecies MAbs specific for L2. The EBs were incubated with trypsin, solubilized with SDS and 2ME then separated by PAGE and immunoblotted. The first observation was that there was a specific digestion sequence. Two MOMP peptides of 33 Kd and 35 Kd are immediately seen. The 35 Kd peptide has all three epitopes while the 33 Kd fragment carries only the type specific and the UM-3 epitope. This means the UM-1 epitope is on either a 2 Kd or 7 Kd fragment which is now one end of the MOMP molecule. An alternative explanation is the UM-1 epitope is present on the larger fragments but has

been inactivated by the trypsin treatment. The 33 Kd peptide continues to be digested and losses a functional UM-3 epitope until a 25 Kd peptide remains which has only the type specific epitope. Within the time period of the experiment (8 hours), the 25 Kd fragment is stable suggesting it is hidden or protected from further attack by the trypsin. It appears that there are four trypsin sites that are surface exposed. The sequential digestion shows that there are two equally accessible sites which when cleaved, probably allow an opening of the MOMP or the membrane such that the two other sites can be accessed, however, there comes a point when the trypsin cannot reach any further sites.

There appeared to be a number of small peptides produced so we wondered if they were being released from the membrane. The procedure here was to centrifuge the digested EBs and look at both the pellet and the supernatant with silver stain and immunoblot. Other than the soluble trypsin molecule, there were no peptide fragments seen on the silver stain and there was nothing reactive with the immunoblotting using the MOMP MAbs. Because the chlamydial membrane is felt to be held together by inter- and intra-molecular disulfide bonding, we added DTT to the digestion mix to try and release small peptides. Again, no peptides were seen either by silver staining or immunoblotting. This would suggest that even though any disulfide bonds are broken, the peptides may be imbedded in the membrane and are unable to be released. Interestingly, a number of other antigens are released by the DTT treatment but the MOMP appears unaffected.

The information to date concerning MOMP peptides has used MOMP cut from gels or purified by column chromatography. This allowed comparison of peptide maps between serovars but gave no specific information pertaining to surface exposed portions of the protein. We took advantage of the ability of the chlamydial outer membrane to remain intact after trypsinization

to isolate a number of MOMP peptides cleaved at surface accessible areas. With serovar D, there are a number of surface exposed trypsin sites but two are specifically attacked compared to the others. Cleavage at the first site results in a 17 Kd amino terminal peptide and a 26 Kd peptide while the second site gives two peptides of about 20 Kd each. We were able to obtain amino acid sequence data on the 17 Kd and 26 Kd peptides which compared favourably with the published sequences for serovars L_2, B and C (Stephens et al, 1987).

Sequence data proved that the 17 Kd peptide is at the carboxyterminus of MOMP and that the 26 Kd peptide results from a trypsin site at amino acid number 147. The two peptides at MW 20 Kd suggested another trypsin site around position 190. There is a trypsin site at position 147 for serovar B while sites at positions 152 and 142 are found with serovars L_2 and C, respectively. There is a conserved trypsin site for serovars L_2, B and C at position 200 which is close to the predicted value of 190 for serovar D.

This method of producing MOMP peptides also lent itself to HPLC separation as only a few peptides were produced and the sample is very clean of cytoplasmic proteins. One small peptide isolated by HPLC was sequenced and was found to be cleaved at amino acid number 310. There is a trypsin site present in the same position with serovars L_2 and B with a site nearby at position 307 for serovar C. Thus, this site may be surface exposed on D MOMP as well.

The protease action causes a loosening of the membrane but no release of the MOMP which, on the whole, is protected from the proteases. With all this adjustment taking place in the membrane, we wanted to see the effect on infectivity. Hackstadt and Caldwell (1985) had previously found no detrimental effects on the infectivity. We took samples from each time

period using three different amounts of trypsin and used the EBs to infect HeLa cell monolayers. I think to everyone's surprise, there was an increase in infectivity of the treated EBs compared to the untreated controls. This increase was transient lasting up to two hours of trypsin incubation at which time the infectivity fell below that of the controls. There was no apparent correlation between the increased infectivity and the stage of MOMP degradation or any of the other antigens we followed. It may be that the protease action triggers some process which then results in greater infectivity of the EBs. This would also call to question the role of protein ligands for the eucaryotic cell on the EB as protease action should, theoretically, decrease infectivity rather than enhance.

Hackstadt et al (1985) looked at the effect of protease inhibitors on chlamydial infectivity. He found that two inhibitors of trypsin and chymotrypsin abolished infectivity while other inhibitors were shown to have no effect. This could suggest that the EB might be enzymatically "activated" and that in our case by adding the trypsin we are helping the process along. However, a point is reached when the trypsin starts to degrade important functional proteins bringing on a decrease in infectivity. The question is whether the hypothetical enzyme is supplied by the EB or is naturally occurring on the eucaryotic cell surface or is found perhaps inside the cell. Newhall (1987) has postulated an intracellular cross linking mechanism which may be enzymatic to hook the various outer membrane molecules together by disulfide bonds. Maybe there is a similar enzyme which helps break the bonds to encourage nutrient uptake through the MOMP porins eventually leading to reorganization into the RB.

SUMMARY

The data compiled during the course of this thesis has allowed us to make a number of important observations. Using MAbs, we identified two new subspecies epitopes on the MOMP common to serovars G, C, F and L2, G, E, MoPn, which have not been previously described. This information adds to the knowledge on the relatedness of the various serovars and establishes another link between the C.trachomatis serovars and the MoPn strain which are not thought to be closely related.

We were able to show that four antigens are immunoaccessible at the surface of the intact EB. These are the MOMP, LPS, 75 Kd antigen and 29 Kd antigen. The first two have been previously described, while the second two are new additions to the group of macromolecules which project out from the chlamydial outer membrane. Five other antigens of MW 70 Kd, 60 Kd, 57 Kd, 32 Kd and 17 Kd, while not surface exposed, are found in the outer membrane since they were accessible to proteolysis. The majority of the above antigens were shown to be recognized by the human immune system during a natural chlamydial infection providing more evidence that these antigens are on, or near, the surface of the EB.

The MOMP, LPS, 75 Kd, 70 Kd and 17 Kd antigens were found to be genus specific antigens common to the C.trachomatis serovars and the C.psittaci MnPn strain. The other antigens of MW 60 Kd, 57 Kd, 32 Kd and 29 Kd were species specific, common only among the C.trachomatis serovars.

While many antigens induce an antibody response during natural infection, we were able to show that only antibodies directed against the MOMP and 75 Kd antigens neutralized in vitro C.trachomatis infection of HeLa cells.

Our procedure of protease treatment of the intact EB followed by isolation of the outer membrane complex allowed the specific identification of

the surface exposed regions of the MOMP. These results suggested that for serovar D sites at amino acid 147 and amino acid 310 are most accessible to trypsin. An unexpected off-shoot of this work was the observation that protease treatment of whole EBs enhanced infectivity. We may be enhancing or mimicing a natural process which allows a better chance for successful infection of the cell by the EB. If we could understand what is happening at this stage, perhaps by reversing the process, infection of the cell could be prevented.

REFERENCES

Aebersold, R.H., D.B. Teplow, L.E. Hood, and S.B.H. Kent. 1986. Electroblotting onto activated glass. J. Biol. Chem. 216:4229-4238.

Allan, I., and J.H. Pearce. 1983. Differential amino acid utilization by Chlamydia psittaci (strain guinea pig inclusion conjunctivitis) and its regulatory effect on chlamydial growth. J. Gen. Microbiol. 129:1991-2000.

Allan, I., and J.H. Pearce. 1983. Amino acid requirements of strains of Chlamydia trachomatis and C.psittaci growing in McCoy cells: Relationship with clinical syndrome and host origin. J. Gen. Microbiol. 129:2001-2007.

Allan, I., T.M. Cunningham, and M.A. Lovett. 1984. Molecular cloning of the major outer membrane protein of Chlamydia trachomatis. Infect. Immun. 45:637-641.

Allan, I., and J.H. Pearce. 1986. Association of Chlamydia trachomatis with novel cultured cells. In: Oriel, D., G. Ridgway, J. Schachter, D. Taylor-Robinson, and M. Ward (Eds.), Chlamydial Infections. Proceedings of the 6th International Symposium on Human Chlamydial Infections, Sanderstead, Surrey, June 15-21, 1986; Cambridge University Press, London. Pp. 35-38.

Barbour, A.G., K. Ichiamans, T. Hackstadt, L. Perry, and H.D. Caldwell. 1982. Chlamydia trachomatis has penicillin-binding proteins but not detectable muramic acid. J. Bacteriol. 151:420-428.

Barenfanger, J., and A.B. MacDonald. 1974. The role of immunoglobulins in the neutralization of trachoma infectivity. J. Immun. 113:1607-1617.

Barnes, R.C., S.-P. Wang, C.-C. Kuo, and W.E. Stamm. 1985. Rapid immunotyping of Chlamydia trachomatis with monoclonal antibodies in a solid-phase enzyme immunoassay. J. Clin. Microbiol. 22:609-613.

Batteiger, B., W.J. Newhall, and R.B. Jones. 1982. The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. J. Immunol. Methods 55:297-307.

Batteiger, B.E., W.J. Newhall, and R.B. Jones. 1985. Differences in outer membrane proteins of the lymphogranuloma venereum and trachoma biovars of Chlamydia trachomatis. Infect. Immun. 50:488-494.

Batteiger, B.E., W.J. Newhall, P. Terho, C.E. Wilde, and R.B. Jones. 1986. Antigenic analysis of the major outer membrane protein of Chlamydia trachomatis with murine monoclonal antibodies. Infect. Immun. 53:530-533.

Batteiger, B.E., and R.G. Rank. 1987. Analysis of the humoral immune response to chlamydial genital infection in guinea pigs. Infect. Immun. 55:1767-1773.

Bavoil, P., A. Ohlin, and J. Schachter. 1984. Role of disulfide bonding in outer membrane structure and permeability in Chlamydia trachomatis. Infect. Immun. 44:479-485.

Becker, Y., H. Loker, I. Sarov, Y. Asher, B. Gutter, and Z. Zakay-Rones. 1971. Studies on the molecular biology of trachoma agent. In: R.L. Wichols (Ed.), Trachoma and Related Disorders Caused by Chlamydial Agents. Excerpta Medica, Royal Van Gorcum, Assen. pp. 13-26.

Blyth, W.A., P. Reeve, D.M. Graham, and J. Taverne. 1962. The production of antisera that neutralize Blennorrhoea virus. Br. J. Exp. Pathol. 43:340-343.

Bose, S.K., and R.G. Paul. 1982. Purification of Chlamydia trachomatis lymphogranuloma venereum elementary bodies and their interaction with HeLa cells. J. Gen. Microbiol. 128:1371-1379.

Bose, S.K., G.B. Smith, and R.G. Paul. 1983. Influence of lectins, hexoses, and neuraminidase on the association of purified elementary bodies of Chlamydia trachomatis UW-31 with HeLa cells. Infect. Immun. 40:1060-1067.

Bose, S.K., and P.C. Goswami. 1986. Chlamydial adherence to glycosylation-defective mutants of Chinese hamster cell lines. Red Book, pp. 31-34.

Brade, L., M. Nurminen, P.H. Makela, and H. Brade. 1985. Antigenic properties of Chlamydia trachomatis lipopolysaccharide. Infect. Immun. 48:569-572.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of proteindye binding. Anal. Biochem. 72:248-254.

Brunham, R.C., C.-C. Kuo, L. Cles, and K.K. Holmes. 1983. Correlation of host immune response with quantitative recovery of Chlamydia trachomatis from the human endocervix. Infect. Immun. 39:1491-1494.

Brunham, R.C., I.W. Maclean, B.A.O. Binns, and R.W. Peeling. 1985. Chlamydia trachomatis: Its role in tubal infertility. J. Infect. Dis. 152:1275-1282.

Brunham, R.C., R. Peeling, I. Maclean, J. McDowell, K. Persson, and S. Osser. 1987. Postabortal Chlamydia trachomatis salpingitis: Correlating risk with antigen specific serologic responses and with neutralization. J. Infect. Dis. 155:749-755.

Byrne, G.I. 1978. Kinetics of phagocytosis of Chlamydia psittaci by mouse fibroblasts (L cells): Separation of the attachment and ingestion stages. Infect. Immun. 19:607-612.

Byrne, G.I., and J.W. Moulder. 1978. Parasite-specified phagocytosis of Chlamydia psittaci and Chlamydia trachomatis by L and HeLa cells. Infect. Immun. 19:598-606.

Caldwell, H.D., C.-C. Kuo, and G.E. Kenny. 1975a. Antigenic analysis of Chlamydiae by two-dimensional immunoelectrophoresis. I. Antigenic heterogeneity between C.trachomatis and C.psittaci. J. Immunol. 115:963-968.

Caldwell, H.D., C.-C. Kuo, and G.E. Kenny. 1975b. Antigenic analysis of Chlamydiae by two-dimensional immunoelectrophoresis. II. A trachoma-LGV-specific antigen. J. Immunol. 115:969-975.

Caldwell, H.D., and C.-C. Kuo. 1977a. Purification of a Chlamydia trachomatis-specific antigen by immunoadsorption with monospecific antibody. J. Immunol. 118:437-441.

Caldwell, H.D., and C.-C. Kuo. 1977b. Serologic diagnosis of lymphogranuloma venereum by counter immunoelectrophoresis with a Chlamydia trachomatis protein antigen. J. Immunol. 118:442-445.

Caldwell, H.D., J. Kromhout, and J. Schachter. 1981. Purification and partial characterization of the major outer membrane protein of Chlamydia trachomatis. Infect. Immun. 31:1161-1176.

Caldwell, H.D., and J. Schachter. 1982. Antigenic analysis of the major outer membrane protein of Chlamydia spp. Infect. Immun. 35:1024-1031.

Caldwell, H.D., and L.J. Perry. 1982. Neutralization of Chlamydia trachomatis infectivity with antibodies to the major outer membrane protein. Infect. Immun. 38:745-754.

Caldwell, H.D., and R.C. Judd. 1982. Structural analysis of chlamydial major outer membrane proteins. Infect. Immun. 38:960-968.

Caldwell, H.D., and P.J. Hitchcock. 1984. Monoclonal antibody against a genus-specific antigen of Chlamydia species: Location of the epitope on chlamydial lipopolysaccharide. Infect. Immun. 44:306-314.

Caldwell, H.D., S. Stewart, S. Johnson, and H. Taylor. 1987. Tear and serum antibody response to Chlamydia trachomatis antigens during acute chlamydial conjunctivitis in monkeys as determined by immunoblotting. Infect. Immun. 55:93-98.

Chang, J.-J., K. Leonard, T. Arad, T. Pitt, Y.-X. Zhang, and L.-H. Zhang. 1982. Structural studies of the outer envelope of Chlamydia trachomatis by electron microscopy. J. Molec. Biol. 161:579-590.

Clark, R.B., I. Nachamkin, P.F. Schatzki, and H.P. Dalton. 1982. Localization of distinct surface antigens on Chlamydia trachomatis HAR-13 by immune electron microscopy with monoclonal antibodies. Infect. Immun. 38:1273-1278.

Conway, D., C.M.A. Glazener, E.O. Caul, J. Hodgson, M.G.R. Hull, S.K.R. Clarke, and G.M. Stirrat. 1984. Chlamydial serology in fertile and infertile women. Lancet 1:191-193.

Dhir, S.P., S. Hankomori, G.E. Kenny, and J.T. Grayston. 1972. Immunochemical studies on chlamydial group antigen (presence of a 2-keto-3-deoxycarbohydrate as immunodominant group). J. Immunol. 109:116-122.

Eissenberg, L.G., P.B. Wyrick, C.H. Davis, and J.W. Rumpp. 1983. Chlamydia psittaci elementary body envelopes: Ingestion and inhibition of phagolysosome fusion. Infect. Immun. 40:741-751.

Fedorko, D.P., R.B. Clark, I. Nachamkin, and H.P. Dalton. 1987. Complement dependent in vitro neutralization of Chlamydia trachomatis by a subspecies monoclonal antibody. Med. Microbiol. Immunol. 176:225-228.

Friis, R.R. 1972. Interaction of L cells and Chlamydia psittaci: Entry of the parasite and host responses to its development. J. Bacteriol. 110:706-721.

Furness, G., D.M. Graham, and P. Reeve. 1960. The titration of trachoma and inclusion blennorrhoea viruses in cell culture. J. Gen. Microbiol. 23:613-619.

Goldstein, J.L., R.G.W. Anderson, and M.S. Brown. 1979. Coated pits, coated vesicles, and receptor-mediated endocytosis. Nature 279:679-685.

Grayston, J.T., and S.-P. Wang. 1975. New knowledge of chlamydiae and the diseases they cause. J. Infect. Dis. 132:87-105.

Grayston, J.T., C.-C. Kuo, S.-P. Wang, and J. Altman. 1986. A new Chlamydia psittaci strain, TWAR, isolated in actue respiratory tract infections. N. Engl. J. Med. 315:161-168.

Hackstadt, T., and H.D. Caldwell. 1985. Effect of proteolytic cleavage of surface-exposed proteins on infectivity of Chlamydia trachomatis. Infect. Immun. 48:546-551.

Hackstadt, T., W.J. Todd, and H.D. Caldwell. 1985. Disulfide-mediated interactions of the chlamydial major outer membrane protein: Role in the differentiation of Chlamydiae? J. Bacteriol. 161:25-31.

Hackstadt, T. 1986. Identification and properties of chlamydial polypeptides that bind eucaryotic cell surface components. J. Bacteriol. 165:13-20.

Hackstadt, T. 1986. Release of Chlamydiae from the cell surface by heparin and variation of heparin binding proteins between strains. In: Oriel, D., G. Ridgway, J. Schachter, D. Taylor-Robinson, and M. Ward (Eds.), Chlamydial Infections. Proceedings of the 6th International Symposium on Human Chlamydial Infections, Sanderstead, Surrey, June 15-21, 1986; Cambridge University Press, London. Pp. 19-22.

Hatch, T.P. 1975. Competition between Chlamydia psittaci and L cells for host isolenaine pools: A limiting factor in chlamydial multiplication. Infect. Immun. 12:211-220.

Hatch, T.P., D.W. Vance, and E. Al-Hossainy. 1981. Attachment of Chlamydia psittaci to formaldehyde-fixed and unfixed L cells. J. Gen. Microbiol. 125:273-283.

Hatch, T.P., D.W. Vance, and E. Al-Hossainy. 1981. Identification of a major envelope protein in Chlamydia spp. J. Bacteriol. 146:426-429.

Hatch, T.P., I. Allan, and J.H. Pearce. 1984. Structural and polypeptide differences between envelopes of infective and reproductive life cycle forms of Chlamydiae spp. J. Bacteriol. 157:13-20.

Hitchcock, P.J., L. Leive, P.H. Makela, E.T. Rietschel, W. Strittmatter, and D.C. Morrison. 1986. Lipopolysaccharide nomenclature - past, present and future. J. Bacteriol. 166:699-705.

Hodinka, R.L., and P.B. Wyrick. 1986. Ultrastructural study of mode of entry of Chlamydia psittaci into L-929 cells. Infect. Immun. 54:855-863.

How, S.J., D. Hobson, and C.A. Hart. 1984. Studies in vitro of the nature and synthesis of the cell wall of Chlamydia trachomatis. Curr. Microbiol. 10:269-274.

Howard, L.V. 1975. Neutralization of Chlamydia trachomatis in cell culture. Infect. Immun. 11:698-703.

Inman, R.D., M.E.A. Johnston, B. Chin, J. Falk, and M. Petric. 1987. Immunochemical analysis of immune response to Chlamydia trachomatis in Reiter's syndrome and nonspecific urethritis. Clin. Exp. Immunol. 69:246-254.

Judd, R.C., and H.D. Caldwell. 1985. Identification and isolation of surface-exposed portions of the major outer membrane protein of Chlamydia trachomatis by two-dimensional peptide mapping and high-performance liquid chromatography. J. Liq. Chrom. 8:1559-1571.

Kaul, R., and W.M. Wenman. 1985. Cloning and expression in Escherichia coli of a species-specific Chlamydia trachomatis outer membrane antigen. FEMS Microbiol. Lett. 27:7-12.

Kaul, R., and W.M. Wenman. 1986. Cyclic AMP inhibits developmental regulation of Chlamydia trachomatis. J. Bacteriol. 168:722-727.

Kingsbury, D.T., and E. Weiss. 1968. Lack of deoxyribonucleic acid homology between species of the genus Chlamydia. J. Bacteriol. 96:1421-1423.

Kunimoto, D., and R.C. Brunham. 1985. Human immune response and Chlamydia trachomatis infection. Rev. Infect. Dis. 7:665-673.

Kuo, C.-C., S.-P. Wang, and J.T. Grayston. 1973. Effect of polycations, polyanious, and neuraminidase on the infectivity of trachoma - inclusion conjunctivitis and lymphogranuloma venereum organisms in HeLa cells: Sialic acid residues as possible receptors for trachoma-inclusion conjunctivitis. Infect. Immun. 8:74-79.

Kuo, C.-C., and J.T. Grayston. 1976. Interaction of Chlamydia trachomatis organisms and HeLa 229 cells. Infect. Immun. 13:1103-1109.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lawn, A.M., W.A. Blyth, and J. Taverne. 1973. Interactions of TRIC agents with macrophages and BHK-21 cells observed by electron microscopy. J. Hyg. Camb. 71:515-528.

Lee, C.K. 1981. Interaction between a trachoma strain of Chlamydia trachomatis and mouse fibroblasts (McCoy cells) in the absence of centrifugation. Infect. Immun. 31:584-591.

Levy, N.J. 1979. Wheat germ agglutinin blockage of chlamydial attachment sites: Antagonism by N-acetyl-D-glucosamine. Infect. Immun. 25:946-953.

Lucero, M.E., and C.-C. Kuo. 1985. Neutralization of Chlamydia trachomatis cell culture infection by serovar-specific monoclonal antibodies. Infect. Immun. 50:595-597.

Manire, G.P., and A. Tamura. 1967. Preparation and chemical composition of the cell walls of mature infections. Dense forms of meningopneumonitis organisms. J. Bacteriol. 94:1178-1183.

McComb, D.E., R.L. Nichols, D.Z. Semine, J.R. Evrard, S. Alpert, V.A. Crockett, B. Rosner, S.H. Zinner, and W.M. McCormack. 1979. Chlamydia trachomatis in women: Antibody in cervical secretions as a possible indication of genital infection. J. Infect. Dis. 139:628-633.

Monnickendam, M.A., S. Darougar, J.D. Treharne, and A.M. Tilbury. 1980. Develop[ment of chronic conjunctivitis with scarring and panmis, resembling trachoma, in guinea pigs. Br. J. Ophthalmol. 64:284-290.

Morrissey, J.H. 1981. Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. Anal. Biochem. 117:307-310.

Moulder, J.W. 1974. Intracellular parasitism: Life in an extreme environment. J. Infect. Dis. 130:300-305.

Moulder, J.W. 1985. Comparative biology of intercellular parasitism. Microbiol. Rev. 49:298-337.

Murray, A., and M.E. Ward. 1984. Control mechanisms governing the infectivity of Chlamydia trachomatis for HeLa cells: The role of calmodulin. J. Gen. Microbiol. 130:193-201.

Murray, E.S., L.T. Chanbonnet, and A.B. MacDonald. 1973. Immunity to chlamydial infection of the eye. I. The role of circulatory and secretory antibodies in resistance to reinfection with guinea pig inclusion conjunctivitis. J. Immunol. 110:1518-1525.

Nano, F.E., and H.D. Caldwell. 1985. Expression of the chlamydial genus-specific lipopolysaccharide epitope in Escherichia coli. Science 228:742-744.

Nano, F.E., P.A. Barstad, L.W. Mayer, J.E. Coligan, and H.D. Caldwell. 1985. Partial amino acid sequence and molecular cloning of the encoding gene for the major outer membrane protein of Chlamydia trachomatis. Infect. Immun. 48:372-377.

Newhall, W.J., B. Batteiger, and R.B. Jones. 1982. Analysis of the human serological response to proteins of Chlamydia trachomatis. Infect. Immun. 38:1181-1189.

Newhall, W.J., and R.B. Jones. 1983. Disulfide-linked oligomers of the major outer membrane protein of Chlamydiae. J. Bacteriol. 154:998-1001.

Newhall, W.J. 1986. Purification and structural characterization of chlamydial outer membrane proteins. In: Oriel, D., G. Ridgway, J. Schachter, D. Taylor-Robinson, and M. Ward (Eds.), Chlamydial Infections. Proceedings of the 6th International Symposium on Human Chlamydial Infections, Sanderstead, Surrey, June 15-21, 1986; Cambridge University Press, London. Pp. 93-96.

Newhall, W.J. 1987. Biosynthesis and disulfide cross-linking of outer membrane components during the growth cycle of Chlamydia trachomatis. Infect. Immun. 55:162-168.

Nichols, R.L., R.E. Oertley, C.E.O. Fraser, A.B. MacDonald, and D.E. McComb. 1973. Immunity to chlamydial infections of the eye. IV. Homologous neutralization of trachoma infectivity for the owl monkey conjunctivae by eye secretions from humans with trachoma. J. Infect. Dis. 127:429-432.

Nurminen, M., M. Leinonen, P. Saikku, and P.H. Makela. 1983. The genus-specific antigen of chlamydia: Resemblance to the lipopolysaccharide of enteric bacteria. Science 220:1279-1281.

Nurminen, M., K. Lounatmaa, M. Leinonen, and E. Wahlstrom. 1984. The effect of mercerptoethanol on the solubilization of the 39.5 Kdal major outer membrane protein of elementary bodies of Chlamydia trachomatis and purification of the protein. FEMS Microbiol. Lett. 24:185-191.

Nurminen, M., E. Wahlstrom, M. Kleemola, M. Leimonen, P. Saikku, and P.H. Makela. 1984. Immunologically related ketodeoxyoctanate-containing structures in Chlamydia trachomatis, Re mutants of Salmonella species, and Acinetobacter calcoceticus var onitratus. Infect. Immun. 44:609-613.

Nurminen, M., E.T. Rietschel, and H. Brade. 1985. Chemical characterization of Chlamydia trachomatis lipopolysaccharide. Infect. Immun. 48:573-575.

Palmer, L., and S. Falkow. 1986. A common plasmid of Chlamydia trachomatis. Plasmid. 16:52-62.

Palmer, L., S. Falkow, and L. Klevan. 1986. 165 ribosomal RNA genes of Chlamydia trachomatis. In: Oriel, D., G. Ridgway, J. Schachter, D. Taylor-Robinson, and M. Ward (Eds.), Chlamydial Infections. Proceedings of the 6th International Symposium on Human Chlamydial Infections, Sanderstead, Surrey, June 15-21, 1986; Cambridge University Press, London. pp. 89-92.

Pastan, I., and M.C. Willingham. 1983. Receptor-mediated endocytosis: Coated pits, receptosomes and the golgi. Trends Biochem. Sci. 8:250-254.

Patterson, S., J.S. Oxford, and R.R. Dommashkin. 1979. Studies on the mechanism of influenza virus entry into cells. J. Gen. Virol. 43:223-229.

Peeling, R., I.W. Maclean, and R.C. Brunham. 1984. In vitro neutralization of Chlamydia trachomatis with monoclonal antibody to an epitope on the major outer membrane protein. Infect. Immun. 46:484-488.

Perez-Martinez, J.A., and J. Storz. 1985. Antigenic diversity of Chlamydia psittaci of mammalian origin determined by microimmunofluorescence. Infect. Immun. 50:905-910.

Peterson, E.M., and L.M. de la Maza. 1983. Characterization of chlamydia DNA by restriction endonuclease cleavage. Infect. Immun. 41:604-608.

Rabinovitch, M. 1968. Phagocytosis: The engulfment stage. Sem. Hematol. 5:134-155.

Rank, R.G., H.J. White, and A.L. Barron. 1979. Humoral immunity in the resolution of genital infection in female guinea pigs infected with the agent of guinea pig inclusion conjunctivitis. Infect. Immun. 26:573-579.

Rank, R.G., and A.L. Barron. 1983. Humoral immune response in acquired immunity to chlamydial genital infection in female guinea pigs. Infect. Immun. 39:463-465.

Sacks, D.L., and A.B. MacDonald. 1979. Isolation of a type-specific antigen from Chlamydia trachomatis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J. Immunol. 122:136-139.

Salari, S.H., and M.E. Ward. 1981. Polypeptide composition of Chlamydia trachomatis. J. Gen. Microbiol. 123:197-207.

Schaffner, W., and C. Weissmann. 1973. A rapid, sensitive, and specific method for the determination of protein in dilute solution. Anal. Biochem. 56:502-514.

Silverstein, S.C., R.M. Steinman, and Z.A. Cohn. 1977. Endocytosis. Ann. Rev. Biochem. 46:669-722.

Soderlund, G., and E. Kihlstrom. 1983. Attachment and internalization of a Chlamydia trachomatis lymphogranuloma venereum strain by McCoy cells: Kinetics of infectivity and effect of lectins and carbohydrates. Infect. Immun. 42:930-935.

Soderlund, G., and E. Kilhstrom. 1983. Effect of methylamine and monodansylcadaverine on the susceptibility of McCoy cells to Chlamydia trachomatis infection. Infect. Immun. 40:534-541.

Stephens, R.S., M.R. Tam, C.-C. Kuo, and R.C. Nowinski. 1982. Monoclonal antibodies to Chlamydia trachomatis: Antibody specificities and antigen characterization. J. Immunol. 128:1083-1089.

Stephens, R.S., and C.-C. Kuo. 1984. Chlamydia trachomatis species-specific epitope detected on mouse biovar outer membrane protein. Infect. Immun. 45:790-791.

Stephens, R.S., C.-C. Kuo, G. Newport, and N. Agabian. 1985. Molecular cloning and expression of Chlamydia trachomatis major outer membrane protein antigens in Escherichia coli. Infect. Immun. 47:713718.

Stephens, R.S., G. Mullenbach, R. Sanchez-Pescador, and N. Agabian. 1986. Sequence analysis of the major outer membrane protein gene from Chlamydia trachomatis serovar L_2. J. Bacteriol. 168:1277-1282.

Stephens, R.S., R. Sanchez-Pescador, E.A. Wagar, C. Inonye, and M.S. Urdea. 1987. Diversity of Chlamydia trachomatis major outer membrane protein genes. J. Bacteriol. 169:3879-3885.

Stirling, P., I. Allan, and J.H. Pearce. 1983. Interference with transformation of Chlamydiae from reproductive to infective body forms by deprivation of cysteine. FEMS Microbiol. Lett. 19:133-136.

Tamura, A., and G.P. Manire. 1967. Preparation and chemical composition of the cell membrane of developmental reticulate forms of meningopneumonitis organisms. J. Bacteriol. 94:1184-1188.

Tamura, A., A. Tanaka, and G.P. Manire. 1974. Separation of the polypeptides of chlamydia and its cell walls by polyacrylamide gel electrophoresis. J. Bacteriol. 118:139-143.

Tamura, A., and G.P. Manire. 1974. Hemagglutinin in cell walls of Chlamydia psittaci. J. Bacteriol. 118:144-148.

Taylor, H.R., E. Young, A.B. MacDonald, J. Schachter, and R.A. Prendergast. 1987. Oral immunization against chlamydial eye infection. Invest. Ophthalmol. Visual Sci. 28:249-258.

Thornley, M.J., S.E. Zamze, M.D. Byrne, M. Lusher, and R.T. Evans. 1985. Properties of monoclonal antibodies to the genus-specific antigen of chlamydia and their use for antigen detection by reverse passive haemagglutination. J. Gen. Microbiol. 131:7-15.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Soc. 76:4350-4354.

Treharne, J.D., R.S.C. Dwyer, S. Darougar, B.R. Jones, and T. Daghfons. 1978. Antichlamydial antibody in tears and sera and serotypes of Chlamydia trachomatis isolated from school children in southern Tunisia. Br. J. Ophthal. 62:509-515.

Wang, S.-P., and J.T. Grayston. 1982. Micro-immunofluorescence antibody responses in Chlamydia trachomatis infection, A review. In: Mardh, P.-A., K.K. Holmes, J.D. Oriel, P. Piot, and J. Schachter (Eds.), Chlamydial Infections. Proceedings of the 5th International Symposium on Human Chlamydial Infections, Lund, Sweden, June 15-19, 1982; Elsevier Biomedical Press, Amsterdam, The Netherlands. Pp. 301-316.

Wang, S.-P., C.-C. Kuo, R.C. Barnes, R.S. Stephens, and J.T. Grayston. 1985. Immunotyping of Chlamydia trachomatis with monoclonal antibodies. J. Infect. Dis. 152:791-800.

Ward, M.E., and H. Salari. 1982. Control mechanisms governing the infectivity of Chlamydia trachomatis for HeLa cells: Modulation by cyclic nucleotides, prostaglandins and calcium. J. Gen. Microbiol. 128:639-650.

Ward, M.E., and A. Murray. 1984. Control mechanisms governing the infectivity of Chlamydia trachomatis for HeLa cells: Mechanisms of endocytosis. J. Gen. Microbiol. 130:1765-1780.

Ward, M.E., J.D. Treharne, and A. Murray. 1986. Antigenic specificity of human antibody to chlamydia in trachoma and lymphogranuloma venereum. J. Gen. Microbiol. 132:1599-1610.

Watkins, N.G., W.J. Hadlow, A.B. Moos, and H.D. Caldwell. 1986. Ocular delayed hypersensitivity: A pathogenetic mechanism of chlamydial conjunctivitis in guinea pigs. Proc. Natl. Acad. Sci. USA 83:7480-7484.

Watkins, N.G., H.D. Caldwell, and T. Hackstadt. 1987. Chlamydial hemagglutinin identified as lipopolysaccharide. J. Bacteriol. 169:3826-3828.

Weiss, E., S. Schramek, N.N. Wilson, and L.W. Newman. 1970. Deoxyribonucleic acid heterogeneity between human and murine strains of Chlamydia trachomatis. Infect. Immun. 2:24-28.

Wenman, W.M., and R.N. Meuser. 1986. Chlamydia trachomatis elementary bodies possess proteins which bind to eucaryotic cell membranes. J. Bacteriol. 165:602-607.

Williams, D.M., J. Schachter, M.H. Weiner, and B. Grubbs. 1984. Antibody in host defense against mouse pneumonitis agent (murine Chlamydia trachomatis). Infect. Immun. 45:674-678.

Zeichner, S.L. 1983. Isolation and characterization of macrophage phagosomes containing infections and heat-inactivated Chlamydia psittaci: Two phagosomes with different intracellular behaviours. Infect. Immun. 40:956-966.

Zhang, Y.-X., S. Stewart, T. Joseph, H.R. Taylor, and H.D. Caldwell. 1987. Protective monoclonal antibodies recognize epitopes located on the major outer membrane protein of Chlamydia trachomatis. J. Immunol. 138:575-581.

APPENDIX A

1. GKNP (10 x)

NaCl	80.0 g
Glucose	10.0 g
KCl	4.0 g
KH_2PO_4	0.6 g
$Na_2HPO_4.2H_2O$	0.6 g
.2% phenol red	100.0 ml
H_2O	900.0 ml

2. Trypsin (1% Stock Solution)

Trypsin	1.0 g
1x GKNP	100.0 ml
Filter sterilize	

3. HBSS (10x)

Same as GKNP (10x) except add:

$MgSO_4.7H_2O$	2.0 g
$CaCl_2.2H_2O$	1.4 g

4. SPG

Sucrose	75.0 g
KH_2PO_4	0.52 g
Na_2HPO_4	1.22 g
Glutamic acid	0.72 g
H_2O	to 1.0 L (pH 7.5)

APPENDIX A (Continued)

5. PAG Solubilization Buffer

.1 M TRIS, pH 6.8	8.5 ml
Glycerol	1.0 ml
2 mercaptoethanol	0.5 ml
SDS	0.25 g
.1% bromphenol blue	0.1 ml

6. HAT

HT (100x stock)

0.03875 g thymidine (final concentration 1.6×10^{-5} M)

0.1361 g hypoxanthine (final concentration 1×10^{-4} M)

100 ml H_2O

A (1000x stock)

17.6 mg aminopterin (final concentration 4×10^{-7} M)

100 ml H_2O

LIST OF SUPPLIERS

1. Beckman Instruments, Toronto, Ontario, Canada

2. Biorad Laboratories, Mississauga, Ontario, Canada

3. Boehringer Mannheim, Dorval, Quebec, Canada

4. DAKO, c/o Cederland Laboratories, Hornby, Ontario, Canada

5. Gibco Canada, Burlington, Ontario, Canada

6. Miles Scientific, Naperville, Illinois, U.S.A.

7. Pharmacia Canada, Dorval, Quebec, Canada

8. Pierce Chemical Company, Rockford, Illinois, U.S.A.

9. Sigma Chemical Company, St. Louis, Missouri, U.S.A.

10. Squibb Canada Inc., Montreal, Quebec, Canada

11. Syva Company, Kanata, Ontario, Canada