CHLAMYDIA TRACHOMATIS INFECTION OF HELA CELLS: ROLE OF OUTER MEMBRANE PROTEINS

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In Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy

by
Rosanna Wai-Wan Peeling

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ROSANNA WAI-WAN PEELING

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

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This thesis is dedicated to Dr. J.C. Wilt who is to me a true model of excellence as a scientist, educator, and administrator. As a young graduate student in the department, and later as a colleague building the virus unit, I was inspired by his insight, selflessness and wisdom. Above all, he is a friend who has always been there for all of us whenever we needed him.

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ABSTRACT

Chlamydia trachomatis is an obligate parasite of eucaryotic cells. The outer membrane of its infectious extracellular form, the elementary body (EB), is protein rich and specifies important functions in its pathogenesis. To define the role of these outer membrane proteins (OMPs) in eucaryotic cell infection, three approaches were used. First, scanning and transmission electron micrographs of chlamydial infection of HeLa cells were examined to provide ultrastructural information and to define stages of chlamydial infection for subsequent studies.

The second approach used monoclonal antibody mediated neutralization of infectivity to probe structure-function relationships of outer membrane antigens. Optimal conditions for an in-vitro neutralization assay were defined. Ninety-nine bybridomas specifying epitopes on 8 antigens were produced and screened for neutralizing activity. Monoclonal antibodies directed to serovar-specific and subspecies-specific epitopes on the major outer membrane protein (MOMP) neutralized infectivity for HeLa cells. This neutralizing activity correlated with immunoaccessibility of the epitope on native EBs and avidity of the antibody. Neutralization was not due to inhibition of attachment to, or endocytosis into host cells, but likely prevented chlamydial intracellular development. Neutralization in-vitro also correlated with protection of mice from toxic death.

The third approach was to use trypsin and 2-mercaptoethanol to alter outer membrane structure and correlate these changes with altered biologic effects in the otherwise metabolically inert EBs. Limited proteolysis enhanced EB infectivity for HeLa cells. The main targets of trypsinization were MOMP and a 75 kDa OMP. The enhancement was not due to increased attachment or uptake but rather due to increased outer membrane permeability as measured by the incorporation of nucleotide triphosphates for RNA polymerase activity. High resolution ^{31}P-nuclear magnetic resonance studies of the ATPase activity in chlamydiae treated with trypsin or 2-mercaptoethanol provided another indicator of increased outer membrane permeability.

Integrating the results of the immunological and biochemical studies, we tested different monoclonal antibodies for ability to block intracellular activity. The inhibition of RNA polymerase and ATPase activities by a neutralizing monoclonal antibody to MOMP suggests that MOMP is the major porin protein on the chlamydial outer membrane, and that enhancement of outer membrane permeability is an important process underlying chlamydial intracellular development.

INTRODUCTION

Chlamydia trachomatis is an obligate intracellular parasite of eucaryotic cells. It is the cause of a wide variety of ocular, genital, and respiratory diseases ranging from acute infections such as conjunctivitis, urethritis, and cervicitis to more chronic infections such as trachoma and pelvic inflammatory disease. Trachoma is an eye disease which was first described in the Ebers Papyrus in Egypt almost 4,000 years ago. Even today, trachoma remains a leading cause of preventable blindness in the developing world. Worldwide, 400 million people are infected, and as many as 20 million are blinded as a result of trachoma. Genital infection with *C. trachomatis* has covertly emerged as a prevalent and damaging sexually-transmitted disease. Approximately, 40% of women with tubal infertility in Canada today had *C. trachomatis* infection as the cause of their infertility.

Chlamydiae have a unique life cycle characterized by dimorphism. Dimorphism appears as the chlamydial solution to the difficult problem of survival in unstable and perhaps hostile environments both inside and outside host cells. After more than 25 years of dedicated research on chlamydiae, James Moulder concluded that "it is indisputable that they are queer bacteria". (Moulder, 1984) The question he posed was "why have the chlamydiae taken up such a difficult way of life?" (Moulder, 1983).

The challenge to the microbiologist is to unravel the molecular mechanisms that enable a queer bacterium with a difficult way of life to remain such a highly successful human pathogen. Only through a better understanding of the molecular basis of its pathogenesis will it be possible to develop strategies for the control and prevention of C. trachomatis infection.

I. LITERATURE REVIEW

A) Taxonomy

The name Chlamydia is derived from the Greek word "Chlamydis" meaning a cloak or mantle. These microorganisms were formerly considered to be one of the mantle viruses. Although chlamydiae are intermediate between smaller bacteria and larger viruses with respect to size and biological properties, they are undoubtedly bacteria. Early workers confused chlamydiae with viruses because they were filterable and exhibited an obligate intracellular parasitism in eucaryotic hosts. The pioneering work of Bedson and Meyer in the early 1900's, and later that of Stanier, Lwoff and Moulder, showed that chlamydiae multiply by binary fission and possess cell walls like bacteria. The family Chlamydiaceae was initially placed in the order Rickettsiales until it was determined that, unlike the rickettsiae, they do not have arthropods as natural hosts and have a life cycle characterized by dimorphic forms. The order Chlamydiales was therefore introduced in Bergey's Manual in 1971 with a single family, Chlamydiaceae, which is monogeneric. There are two species in the genus Chlamydia: trachomatis from "trachoma", the Greek word for roughness, referring to the condition of the infected eye; and psittaci, meaning parrot, an indication of the origin of this species. Recently, Chlamydia trachomatis has been subdivided into three biovars according to the disease manifestation in the human and mouse host. These are

the lymphogranuloma venereum (LGV), trachoma and mouse biovars.

Although morphologically and developmentally similar, the two chlamydial species are readily distinguished by their sulpha resistance and iodine staining properties. C. trachomatis is sulpha sensitive, accumulates glycogen in its inclusion, and is thus readily detected by iodine staining. C. psittaci is sulfa resistant, does not store glycogen and cannot be detected by iodine staining. The natural host of C. trachomatis is humans. Birds and mammals other than humans are the natural hosts of C. psittaci, but humans can become infected by exposure to infected birds or animals. An exception is the recently described TWAR strain of C. psittaci which apparently exhibits human to human transmission (Kuo et al, 1986). The two species of chlamydia share less than 11% homology by DNA hybridization techniques. The intraspecies homology for C. trachomatis lymphogranuloma venereum and trachoma biovars is close to 100%. The mouse biovar is more distantly related and shares only 30-60% DNA relatedness to the two human biovars (Kingsbury and Weiss, 1968; Weiss et al, 1970).

The genome of C. trachomatis is among the smallest of procaryotic genomes. The chromosome has a molecular mass of 660 megadaltons (Md) which represents about 1 x 10^6 nucleotides in a double stranded circular DNA molecule. This size is approximately one quarter the size of the E. coli genome, and is only slightly larger

than that of mycoplasma. The C. psittaci genome is slightly smaller than that of C. trachomatis. Both species have a 4.4 Md plasmid which is phenotypically cryptic. Palmer and Falkow (1986a) showed that although the plasmids of all C. trachomatis human biovars share homology, those of C. psittaci and the mouse pneumonitis strain of C. trachomatis do not. This pattern of conservation may indicate that the plasmid serves an essential function in determining the host range of different chlamydial species. These authors also used restriction endonuclease cleavage site mapping and DNA sequence analysis of ribosomal RNA genes to study the genetic and evolutionary relatedness of chlamydiae and to other genera of bacteria. They confirmed the close relatedness of C. trachomatis trachoma and LGV biovars. C. psittaci and C. trachomatis biovars show only partial homology. Among non-chlamydial genera, Legionella pneumophila, Vibrio cholerae and V. vulnificus show the greatest degree of evolutionary relatedness (Palmer and Falkow, 1986b).

B) Pathogenesis

1. Strategies exploited by a successful obligate intracelluar parasite

When discussing the pathogenesis of intracellular parasites, their success in achieving a fine ecological balance between parasite and host must be considered. An intracellular parasite must i) possess an efficient means for gaining entry into a host cell without causing

irreparable damage; ii) be able to exploit host cell resources during multiplication and maturation; and iii) be released in a form that allows for survival and easy transit to another susceptible host cell. The processes of attachment and endocytosis are early events in the life cycle that appear particularly crucial to the success of intracellular parasites.

The solutions for attachment and entry seen with viruses are among the easiest to understand. Some viruses attach themselves to ubiquitous receptors such as sialic acid residues on the host cell surface. Others make use of clathrin-coated pits to enter the cell along with growth regulatory molecules or essential nutrients.

Moulder (1975) termed phagocytosis the "Trojan Horse" of intracellular parasitism. Most bacteria exist in the extracellular milieu of a host and thus have evolved surface structures such as capsules to evade host phagocytosis. In contrast, intracellular parasites need to be taken up into the host cell to multiply. Thus they have evolved the ability to turn this host defense system of phagocytosis to their own advantage, a situation analogous to the famous Trojan Horse strategy. Bacteria such as Mycobacterium tuberculosis, Mycobacterium leprae, and Legionella are readily phagocytized and actually multiply freely once within macrophages. Others, such as chlamydiae, have evolved further to actually induce phagocytosis among cells not normally phagocytic in order to ensure their chances of

survival. These so called "non-professional phagocytes" are often epithelial cells found on the mucosal surface of the eye, respiratory, gastrointestinal or reproductive tract. Some parasites such as Toxoplasma gondii expend energy during the cell entry. Chlamydiae appear to trigger host cells to expend energy for phagocytosis, a model of true parasitism.

Once the parasite gains entry to a susceptible host cell, it must evade or resist host intracellular defences. Several mechanisms have evolved. Some intracellular bacteria, such as brucellae and Mycobacterium lepraemurium, have cell walls resistant to hydrolases and are therefore able to freely multiply in phagolysosomes despite the presence of lysosomal enzymes. Toxoplasma gondii blocks phagosome acidification and thereby prevents the activation of lysosomal acid hydrolases (Sibley et al, 1985). Other parasites, such as chlamydiae, prevent fusion of the phagosome with host cell lysosomes, perhaps by altering the phagosomal membrane and thus preventing host cell recognition of the phagosome.

With this general background, the specific strategies that Chlamydia trachomatis exploits during intracellular survival will be discussed.

2. Interaction of Chlamydia trachomatis with host cells

Chlamydiae exist in two morphologically and functionally distinct forms, the extracellular elementary body (EB) and the intracellular reticulate

body (RB). The EB is infective but metabolically inert. Following endocytosis, the EB stays within a membrane bound endosome for 8 to 12 hours while it reorganizes into an RB. The RB is approximately three times the diameter of an EB, and is metabolically active. Chlamydiae are energy parasites in that they are unable to generate ATP. The RB uses host derived ATP to fuel its metabolism. RBs multiply by binary fission to form an inclusion, which is visible by light microscopy. At 40 to 48 hours, the RBs condense into EBs and the mature inclusion is extruded from the host cell, releasing new infectious EBs.

2.i) Attachment

The ability to attach to a host cell is the first important property of any successful obligate intracellular parasite. Early work on the interaction of C. trachomatis with host cells made no clear distinctions between the stages of attachment and phagocytosis. Byrne (1978) was the first to discriminate the stages of attachment and ingestion in studies of the phagocytosis of C. psittaci by mouse fibroblasts (L cells). The importance of separating these two stages of pathogenesis can be appreciated from the results of two recent studies. Bard and Levitt (1985) found that clonally derived leukocytes were actually heterogeneous in their interaction with EBs. Some cells were persistently resistant to EB attachment, some allowed attachment but not ingestion of EBs, others

allowed attachment and ingestion but not multiplication, and others were entirely permissive for productive infection. Kuo et al (1976) showed that EBs attached equally well to HeLa and fetal tonsil cells, but fetal tonsil cells failed to support inclusion formation. From these experiments, it appears that chlamydiae require factors in addition to those which determine host cell adhesion if successful eucaryotic cell infection is to occur.

The two approaches generally used in the study of attachment are to modify surface properties of either the EBs or the host cell and observe the resulting differences in the extent and kinetics of attachment. The effects of modifying either the host or the EB surface by various physical or chemical means are summarized in Tables 1 and 2. From these results, it is possible to speculate on what the chemical nature of the EB ligand(s) and the host receptor molecule(s) might be.

The EB ligand(s) is resistant to proteases and mild detergent action but is adversely affected by heating at $37^{o}C$ for 2 hours and at $56^{o}C$ for 3 minutes. Heat instability is most consistent with the ligand being a protein. UV and methyl methane sulphonate treated EBs attached as well as untreated EBs. Thus, the ligand is not newly synthesized nor does attachment depend on EB viability. Treating EBs with hyperimmune antisera also reduces cell-association. Eissenberg et al (1984) showed that isolated envelopes of C. psittaci EBs attached to L cells and were taken up as effectively as

Table 1

ELEMENTARY BODY MODIFICATION: EFFECT ON ATTACHMENT AND PHAGOCYTOSIS

Treatment	Function	EB Serovar/ Host Cell	Effect	References
37°C, 2 hr	Denature protein	B,L_2/HeLa	↓ Attachment	Kuo and Grayston, 1976
60°C, 3 min	Denature protein	A/McCoy	None	Lee, 1987
		L_2,L_3/HeLa	↓ Attachment	Bose and Paul, 1982
		L_1/L	↓ Attachment	Byrne and Moulder, 1982
UV Methyl methane Sulphonate	Damage DNA	L_1/L A/McCoy	None	Byrne and Moulder, 1978 Lee, 1981
Antiserum	Binding/masking Putative cell receptor	L_1/L	↓*	Byrne and Moulder, 1978
Nonidet P-40	Solubilize lipids	L_1/L	None	Byrne and Moulder, 1978
Proteases	Hydrolyse proteins	A/McCoy	None	Lee, 1981
		L_2,L_3/HeLa		Bose and Paul, 1982
		L_1/L		Byrne and Moulder, 1978

↓ = Inhibition

* Stage at which inhibition occurred was not determined.

Table 2

HOST CELL MODIFICATION: EFFECT ON ATTACHMENT AND PHAGOCYTOSIS

Host Cell	Treatment	Function	Effect		References
			LGV Biovars	Non-LGV Biovars	
McCoy HeLa	DEAE-dextran Poly-L-lysine	Reduces negative charge	None ↓	↑ ↑	Kuo et al, 1973 Lee, 1987 Bose and Paul 1982
	Dextran sulphate Heparin	Increases negative charge	↓	↓	Kuo et al, 1973, 1976 Bose and Paul, 1982
HeLa, L, Primary amnion	Proteases	Hydrolyse surface proteins	↓	↓	Byrne and Moulder, 1978 Kordova and Wilt, 1980 Bose and Paul, 1982 Lee, 1981
McCoy			N.D.	None	
HeLa	Wheat germ	block N-acetyl D-glucosa-mine	None	↑	Bose and Goswani, 1986
McCoy	agglutinin		↓		Soderlund and Kihlstrom, 1983
HeLa	Neuraminidase	Block/ destroy Sialic aicd Residues	None	↓	Levy, 1979 Kuo et al, 1973
	Fetuin Ovomucoid N-acetyl Neuraminic acid		None ↓		Bose and Paul, 1982

↑ Enhancement of cell association, N.D. = not done

↓ Inhibition of cell association

intact EBs. This is consistent with the ligand being an intrinsic surface molecule on EBs.

Byrne (1978) suggested that the host receptor(s) may be a glycoprotein or protein as the receptor is protease sensitive. C. trachomatis and C. psittaci appear to have different requirements for a host carbohydrate residue in the binding process. C. psittaci and LGV biovars of C. trachomatis show undiminished attachment when host cells are pre-treated with wheat germ agglutinin, neuraminidase and fetuin. The non-LGV strains of C. trachomatis appear to attach via sialic acid residues as neuraminidase, fetuin, ovomucoid and N-acetyl neuraminic acid block attachment. Allan and Pearce (1986) speculated that chlamydia may exploit multiple host receptors for attachment. They showed undiminished binding of LGV to mutant mammalian cells lacking N-acetyl glucosamine and of non-LGV C. trachomatis strains to cultured mosquito cells lacking N-acetyl neuraminic acid.

Attachment is enhanced by increasing temperature and by centrifugation. It is not clear what role these physical factors play. They may allow for more co-operative interaction as a result of increased molecular mobility or directional force.

Kraaipoel and Duin (1979) showed that both EBs (serovars D and L_2) and HeLa cells are negatively charged at neutral pH. The isoelectric points are 4.64 and 6.85 respectively. Thus, the enhancement of attachment by treatment of host cell surfaces with

strong polycations such as poly-L lysine and DEAE-dextran and the inhibition of attachment by the polyanion heparin and dextran sulphate strongly suggest that non-specific charge interactions play a dominant part in the attachment of non-LGV C. trachomatis strains. Binding does not appear to be avid, as the majority of EBs seem to be spontaneously dissociable with buffer or neuramindase at 5°C.

Wenman and Meuser (1986) and Hackstadt (1986) were the first to examine attachment at a molecular level. Wenman et al identified an 18 kDa and a 32 kDa protein among C. psittaci and C. trachomatis strains that reacted with ^{125}I-labelled HeLa cell membranes. Both molecules are located in the outer membrane complex, and the 18 kDa protein is surface exposed. Monospecific antisera to these proteins showed specific and cross neutralizing activities. Trypsin treated HeLa membrane did not bind to either the 18 KDa or 32 KDa molecules, suggesting that the host cell receptors are proteins. Hackstadt used Triton X-100 to solubilize eucaryotic cells and identified two polypeptides of C. trachomatis EBs of similar molecular weight to those identified by Wenman et al. He subsequently showed that these polypeptides also bind heparin, which displaces cell associated L_2 EBs.

Information on the mechanism of chlamydial attachment, its molecular basis and role in pathogenesis is still very limited. There appear to be no simple

generalizations. Mechanisms may be different for C. psittaci, LGV biovars and C. trachomatis ocular and genital strains. Their interactions differ with different cells. Hopefully, with more sensitive techniques and more relevant and suitable host models, the mechanisms can be elucidated.

2.ii) Endocytosis

Byrne and Moulder (1978) coined the term "parasite-specified phagocytosis" to describe the remarkable characteristic of chlamydiae that permits EBs to be taken into L cells 10 to 100 times more efficiently than are E. coli or latex beads of similar size.

Endocytosis is a temperature dependent process (Silverstein et al, 1977). EBs can attach to host cell surfaces at temperatures as low as 4°C but ingestion does not take place until a temperature threshold of 18 to 21°C is reached. The optimal temperature is about 37°C (Friis, 1972). Temperature dependence may reflect the fact that endocytosis is an energy-dependent process driven by ATP derived from either aerobic oxidation of tricarboxylic acid cycle intermediates or anaerobic glycolysis. Table 3 summarizes the current state of knowledge regarding endocytosis of EBs into eucaryotic cells.

Heat or UV inactivated EBs or even EB envelopes of C. psittaci are ingested as efficiently as intact EBs, although their intracellular fate differs (Eissenberg et

Table 3

INTRACELLULAR HOST CELL MODIFICATIONS: EFFECT ON ENDOCYTOSIS

Treatment	Function	EB Serovar/ Host Cell	Effect	References
Methylamine monodansylcadaverine amantadine	Inhibit receptor-mediated endocytosis	E,L_1/McCoy	↓	Soderlund and Kihlstrom, 1983
		L_2/HeLa	None	Ward and Murray, 1984
Colchicine	Depolymerize cytoplasmic microtubule assembly	L_2/HeLa	None	Byrne and Moulder, 1978
Vincristine Vinblastine	Depolymerize preformed microtubules	L_2/HeLa	↓	Byrne and Moulder, 1978
Cytochalasin B D	Inhibit microfilament contraction	A/McCoy	None	Lee, 1981
		L_2/L cells and HeLa	↓	Byrne and Moulder, 1978 Sompolinsky and Richmond, 1974
Cycloheximide	Inhibit protein synthesis	L_1/HeLa	↓	Byrne and Moulder, 1982
NaF	Inhibit glycolysis	B,L_2/HeLa	None	Kuo and Grayston, 1976

↓ = Decrease in endocytosis

al, 1983). Heat inactivated EBs fused with lysosomes while EB envelopes or intact EBs remain undigested in a membrane bound vacuole. This suggests that a heat labile component on the EB surface, in addition to affecting attachment may also direct intracellular routing of the endosome.

Although cytochalasin B did not inhibit endocytosis of L_2 into HeLa cells (Ward and Murray, 1984) cytocholasin D reduced uptake by 50%. Cytocholasin B may not be as effective as cytocholasin D in affecting endocytosis when particle size is under 600 nm. EBs are approximately 300 nm in diameter. Vinblastine and vincristine, which depolymerize preformed microtubules reduced endocytosis by about 40%, but colchicine, which alters assembly of monomeric and polymeric forms of tubulin, did not.

Ward and Murray (1984) suggested three potential mechanisms of chlamydial endocytosis: the zipper mechanism of Griffin et al (1975), receptor-mediated endocytosis of Goldstein et al (1979) and Patterson's energy independent process where entry is actually effected by a flow of membrane around the EBs (Patterson et al, 1979). Griffin's model involves the sequential binding of the cell receptors to EB ligands as the cell membrane envelops the particle. Ward and Murray suggested that EBs were internalized via Griffin's zipper mechanism based on their inhibitor studies of cytoskeleton elements involved in phagocytosis.

Ward and Murray presented electron microscopy evidence that serovar L_2 EBs were not taken into HeLa 229 cells by receptor-mediated endocytosis via clathrin-coated pits. Opposing evidence comes from the work of Wyrick et al (1986) and Soderlund and Kihlstrom (1983). Wyrick et al presented electron micrographs of C. trachomatis EBs within clathrin-coated pits in McCoy cells. Soderlund and Kihlstrom, using serovar L_1 in McCoy cells, demonstrated a dose-related decrease in internalization of EBs as well as damage to EB intracellular development when inhibitors of receptor-mediated endocytosis were used. The fact that uptake is rapid and saturable (Ward and Murray, 1984) also suggests a possible specific receptor related endocytosis. But the question of which mechanism best describes endocytosis of chlamydia remains unresolved at present.

Whatever the mechanism, the metabolically inert EBs are unlikely to contribute any energy towards the process. Ward and his colleagues (Ward and Salari 1982, Murray and Ward 1984) showed that adhesion of C. trachomatis (serovars H, J, L_1 and L_2) EBs to the HeLa cells was accompanied by a flux of Ca^{++} across the cell membrane and that infection is enhanced by treatment of host cells with a Ca^{++} ionophore. They speculated on a possible calmodulin mediated endocytosis, as calmodulin inhibitors such as pimozide, trifluoroperazine, chlorpromazine, promethazine, and haloperidol reduced chlamydial infectivity. The inhibitory effect was

reversible and dose-related, and was probably associated with decreased uptake because Ca^{++} regulated enzymes govern microfilament function and membrane fluidity. Young et al (1984) showed that localized cytosolic Ca^{++} gradients may be involved in generating signals necessary for phagocytosis in mouse macrophages. Thus the enhancement of C. trachomatis infectivity by centrifugation may also be explained by Ca^{++} influx into the host cell during centrifugation (Ward and Salari, 1982).

Ward and Salari also showed that in addition to the calmodulin control, prostaglandins and cyclic nucleotides might regulate chlamydial uptake. Cyclic nucleotides exert bidirectional control with guanosine 3':5'-cyclic monophosphate (cGMP) stimulating and adenosine 3':5'-cyclic monophosphate (cAMP) inhibiting chlamydial uptake.

During endocytosis EBs likely "select" host cell plasma membrane constituents. This selection may have important results for the subsequent fate of the EB endosome. The vacuole membrane may be modified for nutrient diffusion or evasion of host cell defenses, and must accommodate the expanding inclusion as the RBs multiply. A H^{+}-ATPase enzyme complex is used by endosomal membranes to acidify the endosome, thereby permitting lysosomal enzyme to function in an acidic pH environment. Whether C. trachomatis usurps this enzyme complex to take up host ATP and other essential nutrients to fuel chlamydial replication is unstudied.

2.iii) Inhibition of Phagolysosomal Fusion

The fate of ingested EBs in both professional and non-professional phagocytes has been extensively studied but most of the work has been restricted to the use of C. psittaci on mouse cells. Results suggest that avoidance of phagolysosomal fusion by C. psittaci was mediated by EB surface structures because heating EBs at 56°C for 30 minutes or antibody treatment of EBs caused lysosomal fusion with the chlamydia laden endosome (Friis 1972, Wyrick et al, 1978). Eissenberg et al (1983) subsequently showed that endocytosed C. psittaci EB envelopes were also not fused with lysosomes. However, heating EB envelopes at 56°C for 15 minutes prior to ingestion resulted in fusion of envelope laden endosomes with lysosomes.

Zeichner (1983) analyzed the protein profiles of macrophage phagosomes containing infectious and heat-activated C. psittaci in an attempt to find differences that could account for the different intracellular fates seen with endosomes containing these different particles. Phagosome membrane proteins were resolved by one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Nine proteins in both phagosomes had similar mobilities, only two of which were found in macrophage plasma membrane proteins. However, each type of phagosome had at least one unique protein. Zeichner was unable to draw any conclusion about the role of these proteins in phagolysosomal fusion and speculated that the factor(s)

responsible for inhibition of phagolysosomal fusion must be intrinsic to the EB surface if one considers that EBs are metabolically inert and fusion takes place within 15 minutes of ingestion. Inhibitors of macromolecular synthesis also fail to block the fusion process. Zeichner also suggested that the factor is likely to be a protein, consistent with its heat inactivation properties. There are analogous viral proteins that inhibit membrane fusion.

Recent electron microscopy work by Yong et al (1986) showed that C. trachomatis EBs were readily fused with lysosomes once ingested by human polymorphonuclear leukocytes in the absence of pretreatment with heat or immune serum. The degradation in peroxidase-positive phagolysosomes left very few survivors based on HeLa cell infectivity assays. In contrast, Pearce and Prain (1986) showed that only about 13% of LGV EBs were fused with lysosomes inside McCoy cells. This increased to 62% if EBs were heated. This may actually reflect the difference between phagocytosis mediated by professional phagocytes such as macrophages and polymorphonuclear leukocytes, and "non-professional" phagocytes such as HeLa or McCoy cells, which are not normally phagocytic.

2.iv) EB to RB reorganization and developmental regulation

Little is known about the intracellular events that occur after EB endocytosis. Electron microscopy shows conversion of EB to RB by about 8 hours

post-infection. What triggers the transformation and what are the biochemical and molecular events that underly this transformation continue to intrigue chlamydial researchers.

The morphological and physiochemical nature of EBs and RBs differ. EBs are 200-400 nm in diameter and are metabolically inert. They are similar to bacterial spores in that they must survive an adverse extracellular environment. An EB consists of an electron dense core of DNA and RNA in a 1:1 ratio, surrounded by a protein rich outer membrane tightly cross-linked by disulphide bonds (Newhall and Jones, 1983; Hatch et al, 1984). This outer membrane presumably provides structural stability in the absence of detectable amounts of peptidoglycan (Garrett et al, 1974; Barbour et al, 1982). EBs are resistant to sonication and variations in osmotic pressure. RBs are about 800-1000 nm in diameter and are very fragile. Unlike EBs, RB outer membranes lack disulphide bonds and are susceptible to osmotic pressure and sonication. RBs contain DNA and RNA in a 1:4 ratio and are metabolically active (Hatch et al, 1984; Schachter and Caldwell, 1980). The structural differences between outer membranes of EBs and RBs have been studied by Hatch et al (1984,1986) and by Newhall (Newhall and Jones, 1983, Newhall, 1987) These investigators found that EBs contain three cysteine-rich outer membrane proteins of 12.5 kDa, 40 kDa, and 57-60 kDa. RBs characteristically lack the 12.5 kDa and 57-60 kDa

proteins and commence synthesis of these cysteine-rich proteins late in the growth cycle. The 40 kDa major outer membrane protein (MOMP) appears to be present in equal amounts in both EBs and RBs. This protein becomes extensively cross-linked to the other cysteine rich proteins through disulphide linkages late in the infection cycle. Hatch et al (1986) noted that the disulphide bonds cross-linking MOMP were reduced within 4 hours after entry of EBs into host cells. The reduction process appeared to be host cell-mediated as it was not inhibited by chloramphenicol which would have prevented synthesis of new chlamydial proteins.

The reduction of disulphide bonds in the EB outer membrane appears to be a critical initial step in EB to RB re-organization. During this process the EB envelop must become permeable to nutrients and nucleotides. Direct evidence of porin function in reduced EBs comes from experimental work of Bavoil et al (1984). They showed that liposomes containing the outer membrane complex possessed porin activity. Porin activity was entirely dependent on reduction. Sarov and Becker (1971) in a related series of observations showed that EBs treated with 2-mercaptoethanol possess DNA dependent RNA polymerase activity. Hackstadt et al (1985) showed that EBs treated with reducing agents exhibited increased rates of glutamate oxidation. They also showed that EBs acquired the tinctorial properties of RBs after reduction although no size increase was evident. The reduction of cysteine-rich outer membrane

proteins of EBs likely increases membrane permeability allowing the initiation of metabolic activity.

In the acquisition of essential nutrients from the host cell a keen competition is set up between the host cell and chlamydiae. Growth of chlamydiae is enhanced by the addition of cycloheximide to cell culture media. This likely serves to inhibit host cell protein synthesis allowing the parasite freer access to the nutrient pool. Hatch (1975) found that under limiting conditions, the addition of isoleucine could activate chlamydiae from a latent to a productive state. Allan and Pearce (1983) found that deprivation of leucine, phenylalanine or valine completely inhibited inclusion formation by chlamydiae. Allan et al (1985) found that deprivation of cysteine did not prevent inclusion formation, but rather impeded transformation of RBs to EBs, an effect likely related to impaired synthesis of the late appearing cysteine-rich outer membrane proteins.

The intracellular developmental regulation of chlamydia is complex and much information is lacking. Undoubtedly, such regulation plays an important role in determining host cell susceptibility, and may have important consequences on the outcome of natural infections such as the establishment of latency, asymptomatic infection, and chronic infection.

C. Biochemistry of Chlamydiae

The outer membrane proteins of chlamydiae play a major role in the control of their metabolism. As described in the previous section, the most important change accompanying the differentiation of chlamydiae from a metabolically inert to an active form is the increase in outer membrane permeability. This increase has been shown to allow the initiation of energy metabolism and RNA synthesis in chalmydiae. (Hatch et al, 1982; Sarov and Becker, 1971).

1. Energy Metabolism

The principal defect in the biochemical makeup of chlamydia is the lack of oxidative activity due to the absence of flavoproteins and cytochromes. Hence, chlamydiae are unable to generate adenosine triphosphate (ATP). Instead, they acquire ATP from the host cell and are thus termed "energy parasites".

Winkler (1976) studied the obligate intracellular parasitic bacterium, Rickettsia prowazeki, and found that it is not passively permeable to adenine nucleotides or small molecules. The ATP required by the bacterium is acquired through a carrier-mediated transport system in exchange for adenosine diphosphate (ADP). This transport system is very specific in its requirements. It required the recognition of an adenine base, a ribose sugar and two or three phosphates to function. Hatch et al (1982) found that RBs of C. psittaci transported ATP and ADP by a similar ATP-ADP

exchange mechanism. RBs selectively transport ATP and ADP but not adenosine monophosphate (AMP), adenosine or other nucleotides. This specificity is similar to the requirements of the rickettsial ADP-ATP translocase.

ATP transported into RBs is hydrolyzed into inorganic phosphate and ADP which is then used for further exchange in the transport system. This ATPase activity appears to be magnesium dependent, oubain resistant and oligomycin sensitive. Hatch et al also demonstrated that in the presence of ATP, lysine transport and intracellular accumulation occur against a concentration gradient. Oligomycin, which inhibits ATPase activity, reduced lysine transport. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone, a proton ionophore which dissipates the membrane potential, caused the leakage of intracellular lysine pools.

Weiss and Wilson (1969) observed that C. psittaci incorporation and metabolism of pyruvate, aspartate, and isoleucine are dependent on exogenous ATP. These results are consistent with the concept that acquisition of host ATP is essential to the initiation of biochemical activities in the developing chlamydiae. ATP hydrolysis probably energizes the cytoplasmic membrane and allows for transport of nutrients into chlamydiae against a concentration gradient.

2. RNA Polymerase Activity

Sarov and Becker (1971) demonstrated that when EBs of C. trachomatis were reduced with 2-mercaptoethanol

and then incubated with all four ribonucleotide triphosphates, limited RNA synthesis occurs. The synthesis is DNA dependent since it is inhibited by actinomycin D. Rifampin, which inhibits initiation of procaryotic RNA synthesis, was found to inhibit RNA synthesis in vitro but only after a lag period of 15 minutes. These investigators proposed that chlamydial RNA polymerase molecules are mostly bound to DNA in an initiated form and are therefore not susceptible to rifampin inhibitors until their dissociation from the DNA template to initiate synthesis of new RNA chains.

The activation of RNA polymerase activity in vitro appears to depend only on the presence of DNA and all four nucleoside triphosphates in an appropriate salt buffer. The treatment of EBs with 2-ME enhances the RNA polymerase activity, probably by allowing increased permeability of the EB outer membrane to nucleotide triphosphates.

D. Structure of chlamydiae

The structure of chlamydia has been studied using both the transmission and scanning electron microscope. These studies have been enhanced by the use of ferritin or gold-labelling of antibodies for specific surface proteins. Information obtained from these immunoelectron microscopy studies complement the antigenic analysis of surface macromolecule structures using monoclonal and polyclonal antibodies and peptide mapping analyses.

1. Ultrastructural morphology

As described previously, the EB is a spherical structure 200-300 nm in diameter with an electron dense core surrounded by a cell envelope that consists of an inner cytoplasmic membrane and a trilaminar outer membrane about 9-10 nm thick. Matsumoto (1979) noted the presence of small hemispherical projections on the convex outer surface of EBs, and hexagonal arrays of subunits with 16 nm periodicity on the inner surface when EBs were examined by freeze etching. Nichols et al (1985) using serovar L_2 cultured in mouse fibroblasts, observed two types of surface projections and noted that they were expressed at different stages of the life cycle. They showed that only a minor fraction of EBs possessed the hemispherical projections described by Matsumoto. More frequently observed were spike-like projections found only in intermediate (RB-like) forms of chlamydiae. The spike-like projections spanned the plasma membrane extending some 70-90 nm of which 25-30nm projected beyond the outer membrane. Matsumoto (1981) isolated C. psittaci inclusions formed in L cells at 20 hours post-infection. Thin sections through these inclusions showed the presence of fine, cylindrical projections 11-13 nm in diameter connecting some RBs and the inclusion membrane. Freeze-replica of the convex surface of the inclusions also showed structures 8-10 nm in diameter, arranged in groupings of siix, each group 40-50 nm apart. Matsumto concluded that these

structures are likely RB surface projections which extend through the inclusion membrane.

The functions of these surface projections are not known but the connection of the spike-like projections to the inclusion membrane suggests a possible role in nutrient uptake by chlamydiae.

Chang et al (1982) examined the hexagonal lattice of outer cell membrane envelopes by electron microscopy optical diffraction and computer processing. The lattice had a six-fold symmetry and was 11 nm thick. The hexagonal unit was 17.5 nm in diameter and composed of six subunits (each with a diameter of 3.5-4.0 nm) surrounding a central depression 10 nm in diameter and 8 nm deep. These structures were observed in both EBs and RBs of C. trachomatis and also during binary fission. This model of the hexagonal lattice is consistent with the hexagonal arrays previously observed by Matsumoto and Higashi (1975) for C. psittaci.

2. Antigenic structure and chemical composition

The first antigen identified in Chlamydiae was a lipid soluble, complement-fixing antigen called the chlamydial group antigen. This antigen is found in both C. psittaci and C. trachomatis and is now recognized as the lipopolysaccharide (LPS) in the chlamydial outer membrane. Chlamydiae possess other antigens which are more restricted in their cross reactivity. The earliest indication of antigenic heterogeneity among isolates of C. trachomatis came from the mouse toxicity

prevention studies (Bell et al, 1959; Wang and Grayston et al, 1963). At least 7 distinct strains were recognized by this method. It was not until the introduction of the micro-immunofluorescence test by Wang and Grayston in 1970 that the antigenic heterogeneity of C. trachomatis was fully recognized. The result of this work was the development of a definitive serologic classification of C. trachomatis into the fifteen distinct serovars. Serovars A, B, Ba, and C belong to the trachoma biovar while serovars D through K are generally recognized as genital strains. There are 3 serovars in the LGV biovar and they are numbered L_1 to L_3 (Wang and Grayston, 1982).

The molecular basis for this classification scheme was unknown until SDS-polyacrylamide gel electrophoresis and immunoblotting showed that the serovar or type specificity was based on antigenic heterogeneity of the major outer membrane protein of chlamydiae.

2.i) The major outer membrane protein (MOMP)

The MOMP of EBs constitutes 60% of the total protein content of the outer membrane. MOMP was first observed for C. psittaci by Tamura in 1974 as a thick band in gel electrophoresis, indicating a molecular weight of approximately 43,700. (Tamura et al, 1974). Caldwell et al (1981) and Hatch et al (1981) independently confirmed the existence of MOMP as a surface exposed component of the outer membrane of EBs of C. trachomatis. In contrast to other major outer

membrane proteins of gram negative bacteria, MOMP was resistant to extraction with SDS, EDTA, and Triton X-100 unless solubilization was accompanied by reduction.

Caldwell et al (1981) purified MOMP using a sequential procedure of anionic detergent extraction and hydroxylapatite chromatography. They produced hyperimmune mouse serum to MOMP extracted from serovar L_2 EBs and found cross-reactivity by micro-immunofluorescence to the B complex serovars but not any other trachoma biovars or C. psittaci. Caldwell and Schachter (1982a) subsequently prepared MOMP from different serovars of C. trachomatis and C. psittaci and found that these MOMPs are antigenically complex and heterogeneous. This heterogeneity was confirmed by two-dimensional peptide analysis (Judd and Caldwell, 1985) and by the studies of the reactivities of monoclonal antibodies to MOMP (Stephens et al, 1982).

In 1986, Stephens et al (1986) reported the cloning and sequencing of the gene for MOMP. The MOMP gene consists of a 1,182 base pair open reading frame that encodes 394 amino acids, preceded by a 22 amino acid long leader sequence. Stephens et al (1987) constructed DNA genomic libraries from serovars L_2, B and C and found that the antigenic diversity of MOMP was found in four variable region domains. Two of these domains were candidates for the putative type-specific antigenic determinant. They also identified a species-specific domain of about 16 amino acids that reacted strongly to polyvalent antiserum and to species-specific and

sub-species specific monoclonal antibodies. The genus specific region was not surface immunoaccessible. Serovar L_2 MOMP has 9 cysteine residues as deduced from the nucleotide sequence. Seven of these are conserved among the three serovars.

2.ii) Lipopolysaccharide (LPS)

The LPS of chlamydia resembles the LPS of gram negative bacteria both chemically and biologically. Work by Grayston's group (Dhir et al, 1971; Dhir et al, 1972) showed that the group specific antigen was heat stable at 100°C for 10 minutes or 80°C for 30 minutes and pronase resistant. The lipid antigen is periodate sensitive and was insoluble in water, acetone, methanol but soluble in benzene, chloroform and diethyl ether. These solubility characteristics resemble those of waxes. The immunodominant group, 2-keto-3-deoxyoctanoic acid (KDO) was extracted by mild acid hydrolysis of the water-soluble polysaccharide extract.

Chlamydial LPS is active in the limulus amoebocyte lysate assay and rabbit pyrogen test (Nurminen et al, 1983). These characteristics are attributable to the lipid A portion of LPS in gram negative bacteria.

Monoclonal antibodies to the LPS made by Caldwell and Hitchcock (1984) and Thornley et al (1985) revealed the presence of 3 antigenic domains on the chlamydial LPS molecule, two of which share homology with the Re-LPS chemotype of Salmonella sp. and other enteric bacteria while the third is unique to chlamydiae.

These determinants have genus specificity and constitute the group antigen responsible for the complement fixation reaction.

The gene encoding the enzyme or enzymes producing the genus-specific epitope has been cloned into E. coli (Nano and Caldwell, 1985). The genus epitope is expressed on the surface of viable E. coli recombinants.

In other bacteria, LPS serves to not only stabilize proteins in the outer membrane but also to give the membrane fluidity. LPS may also play a role in promoting hydrophobic interactions in host cell binding. Little is known about the role of LPS in chlamydial pathogenicity but if one considers the similarities in the pathogenesis of C. trachomatis and C. psittaci, in spite of the tremendous antigenic diversity, LPS as a common antigen may determine a shared virulence.

Recent work using ferritin and colloidal gold particles conjugated to monoclonal antibodies have allowed more definitive identification of macromolecules on the surface of chlamydial envelopes. Clark et al. (1982) showed that ferritin-conjugated neutralizing antibodies distributed homogeneously on the outer membrane while non-neutralizing antibodies had a patchy distribution. Kuo et al. (1987) using colloidal gold-labelled monoclonal antibodies to MOMP, did not confirm these results. They showed that serovar-specific and subspecies-specific epitopes on MOMP are more surface exposed than species-specific epitopes. Surface accessibility for anti-MOMP epitopes

correlated with neutralization. The genus-specific epitope on the lipopolysaccharide is not surface accessible on native EBs although it is highly accessible in the micro-immunofluorescence serologic test.

2.iii) Other Antigens

Besides MOMP and LPS, other chlamydial antigens are readily identifiable by immunoblotting using sera collected from infected humans. The prominent antigens are macromolecules of 57 kDa and 60 kDa and 75 kDa. The function of these proteins in pathogenesis are unknown. Antibody to the 57 Kd protein was frequently found in sera of women with tubal infertility (Brunham et al, 1985). Lack of antibodies to the 57 kDa, 60 kDA and 75 kDa antigens was correlated with a risk of developing salpingitis in a cohort of women with chlamydial cervical infections who underwent therapeutic abortion (Brunham et al, 1987).

Zhang et al. (1987) recently described the isolation of a 14/15 kDa cysteine rich outer membrane protein from C. trachomatis serovars B and L_2. From Mabs produced to it, it was found that this protein had both species-specific and biovar specific antigenic determinants. These epitopes did not appear to be immunoaccessible on native EBs by dot-blot assay. Its function at present is unknown.

E. Antibody-Mediated Neutralization of Infectivity

1. In vitro antibody-mediated neutralization of infectivity

i) Studies using whole sera

Reeve and Graham (1962) were the first to show that infectivity of chlamydia could be neutralized with hyperimmune rabbit serum. They observed that neutralization was independent of the concentration of the organism in that the serum neutralized 3.2×10^4 to 3.7×10^7 inclusion forming units/ml. There was only limited cross-neutralization between trachoma strains from different geographical locations. They also noted that a high concentration of serum was required to prevent heat inactivation of the EBs. They later showed that although neutralization was linear with respect to the log of serum dilution, neutralizing antibodies did not parallel complement-fixing antibodies (Blyth et al, 1962).

Banks et al (1970) used a plaque reduction test to show neutralizing antibodies in chicken antisera raised against avian strains of C. psittaci and LGV strains. Similar to the findings of Reeve and Graham, they found no correlation between neutralization and complement-fixation. They noted a 76% heat inactivation of the inoculum over 2 hours at 37°C. Incubation of the neutralization mixture was best at 4°C overnight for their assay.

Friis (1972) was the first to determine at what stage of the chlamydial life cycle antibody mediated

neutralization of infectivity took place. He incubated C. psittaci EBs with antiserum at 37°C for 30 minutes and then titrated the residual infectivity in a plaque assay in L cells. The antibodies were of low avidity in that neutralization decreased if the neutralization reaction mixture was diluted before inoculation onto L cells. He followed the fate of antibody-coated EBs and found that they were internalized into the host cell but were destroyed by phagolysosomal fusion.

These early studies were useful in establishing the antigenic relatedness among serovars of C. trachomatis and C. psittaci. Generally most studies used sera with low neutralization titers (less than 1:128 in most cases) and there were also problems in variability.

Blyth and Taverne (1974) enhanced neutralization of C. trachomatis infectivity in baby hamster kidney cells using a second antibody to immunoglobulin. They were able to increase neutralizing titres as much as 200-fold. There were some problems with non-specific inactivation using this method. Howard (1975) enhanced the efficiency of the neutralization reaction using guinea pig complement. He again noted that heating 1 hour at 37°C resulted in 50-70% inactivation of the inoculum. He determined that neutralization of C. trachomatis serovar A and B with immune rabbit sera was serovar specific and complement dependent. He also found that there was no apparent correlation between fluorescent antibody titres and neutralization titres. Thus neutralizing antibodies were not readily identified

with any known measures of anti-chlamydial immunity such as complement-fixation or immunofluorescence.

Efforts to determine the stage at which neutralization occurred using whole sera were also inconclusive. Friis (1972) showed that neutralization of C. psittaci infectivity for L cells was due to promotion of phagolysosomal fusion. In contrast, Byrne and Moulder (1978) found that neutralization was associated with decreased phagocytosis using the same system. For C. trachomatis, Ainsworth et al (1979) showed that antibody prevented chlamydial attachment to irradiated McCoy cells. Unlike Reeve and Graham, they found that neutralization varied with concentrations of antigen or antibody. High concentrations of antigen or lower concentrations of antibody decreased neutralization.

ii) Studies using monospecific polyclonal antibodies

The use of neutralizing antibodies to determine structure-function relationships became possible when monoclonal antibodies and monospecific polyclonal antibodies were used in studying the mechanism of neutralization. Levy and Moulder (1982) produced antiserum to the major outer membrane protein of C. psittaci and found that it neutralized infectivity in L cells. They did not, however, determine at which stage of infection neutralization took place. Caldwell and Perry (1982b) also found anti-MOMP antibodies were

neutralizing for C. trachomatis serovar L_2 in HeLa cells. They also found that antibody did not inhibit EB attachment or endocytosis and speculated that neutralization of infectivity was probably the result of MOMP being cross-linked by antibody. Intact IgG, rather than Fab fragments, was required for efficient neutralization. Cross-linking of MOMP probably prevented chlamydiae from reorganizing from EBs to RBs.

Wenman et al (1986) produced antisera to two chlamydial proteins of molecular mass 18 kDa and 31 kDa, which are putative ligands for attachment of EBs on HeLa cells. These antisera neutralized infectivity not only against its homologous serovars L_2 but also against serovar J and C. psittaci meningopneumonitis. Both antibodies inhibited EB-host cell association.

iii) Studies using monoclonal antibodies

The only neutralizing monoclonal antibodies (Mabs) identified so far are those directed to epitopes on MOMP. Clark et al (1982) produced Mabs to outer membrane proteins of serovar A. Mabs were identified as type-, subspecies- or species-specific by immunofluorescence in McCoy cells. They found that only type-specific Mabs were neutralizing and that neutralization was complement dependent. Peeling et al (1984) identified a species-specific Mab to MOMP that neutralized in the absence of complement. Similar to the findings of Caldwell and Perry, they found that neutralization was an intracellular event. In contrast,

Lucero and Kuo (1985) found that 5 of 7 type-specific and 2 of 5 subspecies specific Mabs were neutralizing while no species-specific Mab or LPS Mabs neutralized. Like Clark et al, they found neutralization was complement dependent.

These data on neutralization are confusing. They are summarized in Table 4a and 4b. The consensus appears to be that apart from the 18 kDa and 31 kDa proteins, MOMP is the main target for neutralization of infectivity. There are many variations in the neutralization assay itself with respect to time and temperature of incubation, volume of reaction, use of complement, and concentrations of antigens and antibody. Under such conditions, a 50% endpoint may vary depending on conditions used making it difficult to draw conclusions on structure-function relationships using antibody-mediated neutralization of infectivity as a probe.

TABLE 4A

NEUTRALIZATION USING SERA TO C. TRACHOMATIS EBS

Chlamydiae Strain	/Host Cell	Incubation Conditions*	Mechanism of Neutralization	Reference
T'ang (trachoma)	HeLa	37°C/30'	N.D.	Reeve & Graham, 1962
LBl	HeLa	37°C/30'	N.D.	Blyth et al, 1962
Avian C. psittaci & LGV	L cells	4°C/ Overnight	Phagolysosomal fusion	Banks et al, 1970
C. psittaci	L cells	37°C/30'	N.D.	Friis, 1972
Tric	Baby Hamster Kidney	35°C/30'	N.D.	Blyth & Taverne, 1974
A,B	Baby Hamster Kidney	37°C/30'	N.D.	Howard, 1975
C. psittaci	L cells	37°C/60'	Decreases phagocytosis	Byrne & Moulder, 1978
C. trachomatis	McCoy	37°C/15'	Inhibit attachment	Ainsworth et al. 1979

*All reactions were complement independent
N.D. = Not done

TABLE 4B

NEUTRALIZATION USING MONOSPECIFIC POLYCLONAL SERUM

AND MONOCLONAL ANTIBODIES

Chlamydial Strain	Antibody Specificity	Host Cell	Incubation Conditions	Complement Effect	Mechanism of Neutralization	Reference
Polyclonal Serum						
C. psittaci	MOMP	L cells	37°C/60'	Not Used	N.D.	Levy & Moulder, 1982
L_2	MOMP	HeLa	37°C/30'	Enhances	Intracellular	Caldwell & Perry, 1982
L_2	31 kDa 18 kDa	HeLa	37°C/30'	Not Used	Inhibit Attachment	Wenman et al, 1986
Monoclonal Antibodies						
Serovar A	OMP Type sp.	McCoy	37°C/30'	Absolute dependency	N.D.	Clark et al, 1982
L_2	MOMP Sp. sp.	HeLa	37°C/30'	Enhances	Intracellular	Peeling et al, 1984
B,H,I,K	MOMP Type sp.	HeLa	37°C/60'	Absolute dependency	N.D.	Lucero & Kuo, 1985
L_1,L_2,L_3	Sub sp. sp.					

MOMP = Major outer membrane protein
Type Sp = Type specific
Sp sp = Species specific
Sub sp. sp. = Subspecies specific
N.D. = Not done

III. PROPOSAL OF STUDY

The outer membrane of Chlamydia trachomatis is protein rich and is believed to specify essential functions including attachment, endocytosis and inhibition of phagolysosomal fusion. The outer membrane is also a permeability barrier apparently regulated by oxidation-reduction of disulphide bonds.

We are interested in the role of outer membrane proteins (OMPs) in the molecular pathogenesis of C. trachomatis eucaryotic cell infection. We propose to study the role of OMPs in infection using three approaches.

The first is visualization of the life cycle of C. trachomatis using transmission and scanning electron microscopy. This will provide ultrastructural information concerning chlamydiae host cell interaction during different stages of the chlamydial life cycle.

The second approach is to develop monoclonal antibodies to epitopes on outer membrane proteins of C. trachomatis. Optimal conditions for an in vitro neutralization assay will be established. Monoclonal antibodies will be screened for neutralization of chlamydial infectivity. Determination of the antigenic specificity and mechanism of neutralization will be useful in defining structure-function relationships of outer membrane protein antigens.

Since some of the outer membrane proteins of chlamydia are cysteine-rich, our third approach will be modification of the outer membrane of chlamydiae with a

protease and a reducing agent. We will correlate structural changes with changes in infectivity and membrane permeability. These studies will be important in delineating the molecular and biologic effects of changes in chlamydial membrane permeability.

The information gained from these three approaches will be synthesized to provide additional insights into the role of outer membrane proteins in the pathogenesis of Chlamydia trachomatis eucaryotic cell infection.

IV. MATERIALS AND METHODS

A. Preparation of Chlamydia Stock

1. Source 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 (UW43/Cx), I (UW12/Ur), J (UW36/Cx), K (UW31/Cx) and L2 (434/Bu) (courtesy of C.-C. Kuo, University of Washington, Seattle). Chlamydia trachomatis mouse pneumonitis (MoPn) (Weiss strain) and C. psittaci meningopneumonitis (CpMn) strains were kindly provided by J. Schachter (University of California, San Francisco) and W. Wenman (Univerity of Alberta, Edmonton), respectively.

2. Growth of Chlamydia

Chlamydial stocks were grown in HeLa 229 cells which were routinely maintained in the laboratory in Eagle's Minimal Essential Medium with 10% fetal calf serum (FCS) and 1mM glutamate (complete MEM). Each chlamydial strain was inoculated onto 24-hour-old monolayer cultures of HeLa 229 cells in 80 or 175 cm^2 flasks. For serovars A-K, the monolayers were pretreated with DEAE-dextran (30 ug/ml) (Pharmacia, Dorval, Quebec) for 20 minutes at room temperature. The inoculum was allowed to adsorb for 2 hours at room temperature. The monolayer was then rinsed once with Hanks' Balanced Salt Solution (HBSS) and incubated with complete MEM containing 25ug/ml gentamycin, 100 ug/ml vancomycin and 1 ug/ml cycloheximide. C. trachomatis serovars were

incubated for 72 hours at 35°C before harvesting, while C. psittaci and L_2 were incubated for 48 hours at 37°C. One flask with 90-100% infection could be used to infect 6-8 flasks of 24 hour old HeLa 229 monolayers for all C. trachomatis serovars except L_2, which could be passed to 10-15 flasks to yield 90-100% infection per flask.

To harvest the mature infected cultures, the growth medium was discarded and cells were rolled off the flask with approximately 30 glass beads in 10 ml of cold HBSS. The flask was rinsed with an additional 10 ml of cold HBSS. The cell suspension was sonicated on ice in a Branson Sonifier (Danbury, Connecticut) at an output of approximately 25 W for 35 seconds and then centrifuged at 500 g for 15 minutes at 4°C. The pellet containing cell debris was discarded. The supernatant containing EBs and RBs was centrifuged at 30,000 g for 30 minutes at 4°C. The supernatant was discarded and the pellet containing chlamydiae was resuspended in sucrose-phosphate-glutamate buffer (SPG), pH 7.4, using a blunted spinal tap needle. This was used as inoculum for infecting more flasks or aliquoted and frozen at -70°C as stock.

3. Purification of Chlamydia

To purify EBs or RBs from the flask cultures, the same harvesting procedure as previously described was used until the cell debris was discarded after the first centrifugation. Then the supernatant containing chlamydial EBs and RBs was layered on top of an 8 ml

cushion of 35% Renografin (Renografin 76, Squibb, Canada) in HEPES buffer (0.01 M N-2-Hydroxyethylpiperazine-N1-2-ethanesulfonic acid in 0.15 M NaCl) and centrifuged at 43,000 g at 4°C for 60 minutes in a SW27 rotor in the ultracentrifuge. The pellet containing EBs and RBs was resuspended in SPG and layered on top of a discontinuous gradient of 40-52% Renografin in Hepes buffer and centrifuged at 45,000 g for 90 minutes at 4°C in a SW27 rotor in the ultracentrifuge. The EBs form a discrete band at the interface between the 44% and 52% renografin zones while the RBs form a band just below the 40-44% interface. The bands were harvested by suction with a Pasteur pipette, washed twice in HBSS and resuspended in SPG. Purity of the preparation was checked by examination in electron microscopy using negative staining with 1% phosphotungstic acid (PTA), pH 7.0.

4. Quantitation of Chlamydia

i) Particle counting by electron microscopy

Particle counts were made by centrifuging 45 ul of pure EB suspension onto a formvar coated copper grid in a Beckman airfuge for 30 minutes. If necessary, dilutions were made in 1% gluataraldehyde in phosphate-buffered saline (PBS). The grid was stained with 1% phosphotungstic acid (PTA) pH 7.0 and 10 grid squares were counted.

ii) Quantitation by protein content

The protein content of purified EB preparations was determined by two assays.

The Bradford method (Bradford, 1976) was designed for the estimation of soluble proteins at concentrations of 1-25 ug/ml using bovine serum albumin (BSA) as a standard. 200 ul of the dye reagent concentrate (Bio-Rad) was added to 800 ul of the protein solution to be assayed. The mixture was vortexed and allowed to stand at least 5 minutes before measuring the optical density (O.D.) at 595 nm. The reagent was diluted similarly with the buffer used to resuspend the protein, to give a blank solution. A standard curve was constructed from the O.D. of various dilutions of the BSA standard and the protein content of EBs was estimated from the curve using the observed O.D. value.

For particulate protein suspensions and proteins solubilized in detergents such as SDS, a different assay was necessary. This assay is based upon the quantitative binding of amido black to protein immobilized on a nitrocellulose membrane (Schaffner et al, 1973).

The protein to be estimated was diluted with 220 ul of distilled water and then 30 ul of 1 M Tris/2% SDS, pH 7.5, was added. The mixture was vortexed. 50 ul of 90% trichloroacetic acid (TCA) was added, the mixture was vortexed and allowed to sit for at least 2 minutes. The suspension was filtered through a nitrocellulose membrane (0.45 um) in a dot-blot apparatus (Bio-Rad).

The tube was rinsed with 0.4 ml of 6% TCA and this was added to the filter apparatus. The membrane was stained with 0.25% naphthol blue black in 50% methanol, 10% acetic acid for 15 minutes. It was rinsed with water for 45 seconds and then destained with 3 rinses of 90% methanol 2% acetic acid. The spots were blotted dry, cut out, and the dye was eluted with 1 ml of 50% ethanol/25mM NaOH/50mM EDTA for 5 minutes. The absorbance at 620 nm was read with BSA as a standard.

iii) Quantitation by Inclusion Counting

Chlamydia was also quantitated by counting the number of viable elementary bodies that formed inclusions in eucaryotic host cells. The EBs in SPG were inoculated onto 24 hour old HeLa cell monolayers in 1 dram vials containing 12 mm coverslips and allowed to adsorb for 2 hours at room temperature. For trachoma biovars, monolayers were pretreated with DEAE-dextran (30 ug/ml) for 20 minutes. After adsorption, the inoculum was removed and the monolayer washed once with HBSS. The cultures were then incubated at 35°C (or 37°C for C. psittaci and LGV biovars) for 48-72 hours in complete MEM with cycloheximide. At the end of the incubation period, the monolayers were washed once with PBS before being fixed in absolute methanol for 15 minutes. The monolayers were stained for 30 minutes at 37°C with a fluorescein-conjugated monoclonal antibody for C. trachomatis (Syva Microtrak, Syva Co., Palo Alto, CA) or a mouse monoclonal antibody for both C. psittaci

and C. trachomatis and then with fluorescein-conjugated goat anti-mouse immunoglobulins. The number of fluorescent inclusions in 30 high power (40x) fields per coverslip monolayer were counted in a Leitz fluorescent microscope and then converted to number of inclusion-forming-units (IFUs) per coverslip.

3. Electron Microscopy Methods

1. Preparation of Chlamydial Cultures

Twelve mm glass coverslips were seeded with 4 x 10^5 HeLa 229 cells 24 hours prior to infection. The monolayers were infected with serovar J (UW 36/Ur) at a multiplicity of infection of 10. At hourly intervals for the first eight hours and at 12, 24, 36, 48, 75 and 96 hours post infection, coverslips were processed for fixation and dehydration in preparation for scanning or transmission electron microscopy. Coverslips for transmission electron microscopy were pretreated with a light coating of teflon spray and soaked in 10 ug/ml poly-L-lysine solution for an hour before use in cell culture.

2. Fixation and Dehydration

Coverslip cultures were washed twice by immersion in 0.05 M sodium cacodylate washing buffer, pH 7.4, containing 0.01 M $CaCl_2$ and 0.25 M D-glucose. They were then fixed with 2.5% glutaraldehyde in 0.05 M sodium cacodylate for ten minutes. After washing twice with cacodylate washing buffer, the coverslips were fixed

with 1% osmium tetroxide in 0.05 M cacodylate buffer for 20 minutes and then washed and stained with saturated aqueous uranyl acetate for 10 minutes. The coverslips were dehydrated through graded series of acetone concentrations from 10% to 100% for varying times prior to further processing.

3. Scanning Electron Microscopy (SEM) Processing

The fixed and dehydrated coverslip cultures were dried in CO_2 by the critical point drying method. They were mounted on slabs and coated with gold palladium before being examined in a JEOL scanning electron microscope.

4. Transmission Electron Microscopy (TEM) Processing

The coverslip cultures were infiltrated through a graded acetone-DER 332-732 epoxy series for three 30 minute periods and finally in 100% epoxy for 2 hours. The monolayers were initially embedded by placing 2 drops of fresh epoxy upon the coverslips and allowed to polymerize. The glass coverslip was removed from the embedded monolayer by dipping into liquid nitrogen as described by Chang et al. (1971). The embedded monolayer was cut into strips and re-embedded in Beem 00 capsules so that the monolayer was presented for sectioning as cross sections. The sections were stained and examined in a Philips 200 electron microscope.

5. Staining for Coated Pits

The preservation and staining of clathrin-coated membranes in HeLa cells was performed according to the method of Maupin and Pollard (1983) using tannic acid-glutaraldehyde-Saponin fixation. The culture media was replaced with a solution of 1% glutaraldehyde and 2 mg/ml tannic acid in 100 mM sodium phosphate, 50 mM kCl, 5 mM MgCl, pH 7.0 (Buffer A). After fixation at room temperature for 30 minutes, the cells were treated with 1% osmium tetroxide for 20 minutes in buffer A for TEM before dehydration as described.

C. Production of Monoclonal Antibodies

1) Hybridoma Production

i) Injection Schedule

4-6 week old Balb/c female mice were injected with whole elementary bodies of C. trachomatis serovar L_2, J and G in 3 separate fusions. The mice were given intraperitoneally approximately 100 ug of protein in a mixture of 250 ul SPG and 250 ul of Freund's complete adjuvant (Difco). On day 7, they were given the same dose subcutaneously but mixed in Freund's incomplete adjuvant. On day 14 and 21, they were given the EBs in saline intravenously and 3-4 days later, they were bled and their spleens were excised aseptically.

ii) Preparation of Spleen Cells

The hybridoma procedure was adapted from a method described by Stephens et al (1982). The excised immune

spleens were minced in a sterile petri-plate with serum-free RPMI 1640 medium (Gibco). The tissue pieces were triturated with a pasteur pipette to obtain individual cells. The cell suspension was then transferred to a 15 ml conical centrifuge tube and the larger tissue pieces allowed to settle out. The cell suspension was transferred to another conical centrifuge tube and centrifuged at 160 g for 5 minutes at room temperature. The cell pellet was washed once with serum free RPMI and then resuspended in 0.85% ammonium chloride in distilled water for 5' to lyse any erythrocytes in the cell suspension. The pellet was again washed in RPMI and resuspended in RPMI with 10% FCS, and 1 mM sodium pyruvate and 1 mM glutamic acid. The cells were counted by mixing a 50 ul aliquots of cells with 50 ul of 1% trypan blue in 900 ul PBS. 2-6 x 10^8 cells were obtained at each fusion from two mouse spleens.

iii) Preparation of thymocytes

Thymocytes from 3-4 week old Balb/c females were used as feeder cells to maintain the newly fused cells. Thymuses were excised aseptically from the mice and processed using the same protocol as for spleen cells. The yield was usually about 1-2 x 10^7 thymocytes per mouse thymus.

iv) Preparation of mouse myeloma cells

The mouse myeloma cell line used in our fusion was the NS-1 cell line from ATCC. These cells were grown in RPMI 1640 medium with 15% fetal calf serum, 1 mM pyruvate, 100 units/ml Pencillin and 100 ug/ml streptomycin in 7% $C0_2$ at 37°C. The cells were split daily to ensure that they were growing in a log phase for better fusion. Before use in a fusion experiment, live cells were separated from dead cells by centrifugation through Histopaque 1077 (Sigma, St. Louis, MO). Cells from 1-2 flasks of NS-1 cells were pelleted, resuspended in 3 ml of RPMI and layered onto a 3 ml cushion of Histopaque in a 15 ml centrifuge tube. The tube was centrifuged at 200 g for 5 minutes at room temperature. The live cells were harvested from the RPMI/Histopaque interface. The NS-1 cells were washed once and resuspended in RPMI and stained with 1% trypan blue. Viable cells were counted in a hemacytometer.

v) Fusion, growth, and maintenance of hybridomas

When all 3 components had been prepared, the spleen cells were combined with the myeloma cells in a 4 or 5 to 1 ratio to a maximum of 10^8 cells in a 50 ml round bottom glass centrifuge tube. The cell mixture was centrifuged at 160 x g for 5 minutes and the supernatant discarded. The cell pellet was gently resuspended by adding dropwise 1 ml of 40% polyethylene glycol in RPMI (PEG, hybriprep, GIBCO). The fused cells were pelleted by centrifuging at 250 x g for 10 minutes and washed in

10 ml of RPMI. They were centrifuged at 160 x g for 5 minutes to re-pellet the fused cells. The pellet was gently resuspended in 39 ml of RPMI with 15% fetal calf serum and HAT (100 uM Hypoxanthine, 0.4 uM Aminopterin, 16 uM Thymidine). Thymocytes were added at a concentration of 2.5 x 10^6 cells/ml. The final cell mixture was plated onto 96-well flat bottomed microtitre plates at 200 ul/well and incubated at 37°C in 7% CO_2.

The hybridoma cells were fed at 5 day intervals with RPMI and HAT. They were screened for antibody production in the culture supernatant when they approach 50% confluency in the wells.

2. Screening Methods

i) ELISA

Initially, the hybridoma culture supernatants were all screened by an ELISA method. Purified whole EBs were diluted in 0.01 M carbonate-bicarbonate buffer, pH 9.4, to give a concentration of 5-10 ug of protein/ml. 50 ul of this suspension was added to each well of a 96-well u-shaped microtitre plate (Dynatech) and allowed to adsorb overnight at 37°C in a moist chamber. The supernatant was discarded and each well was blocked with 75 ul of 3% bovine serum albumin (BSA) in PBS for 1-2 hours at 37°C. The BSA was discarded and each well was filled with 50 ul of culture supernatants from the hybridoma plates. After incubation at 37°C for 1 hour, the plates were washed 3 times with PBS containing 1% BSA, 0.05% Tween-20. Positive antigen and antibody

reactions were detected by incubating with 1:3000 dilution of horseradish peroxidase (HRP) conjugated rabbit anti-mouse immunoglobulins (Dako Immunoglobulins, Cedarlane Labs, Hornsby, ON) at 37°C for 1 hour. The plates were washed with PBS and developed with 2,2'-azinodi-(3-ethylbenzthiazoline sulfonic acid) (ABTS) (Sigma, St. Louis, MO) made up as a solution of 80 mg per ml in water. 250 ul of ABTS solution was added to 5 ml of 0.1 M citrate buffer, pH 4.2, with 10 ul of 3% hydrogen peroxide added just before use. This was sufficient to develop one plate at 50 ul/well. Positive wells turned dark green while negative ones stayed clear.

Using this method, the clones identified were mainly producing antibodies to MOMP and LPS. This was not surprising as these are expected to be immunodominant molecules on the surface of EBs. In order to identify clones secreting antibodies to other molecules on EBs, seen in human immune responses in natural infections, it was necessary to use immunoblotting as an additional screening method.

ii) Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

The discontinuous buffer system of Laemmli (1970) was adapted in our laboratory for use in the Bio-Rad Protean Dual Slab Gel System. Gels were poured and allowed to set overnight according to Bio-Rad procedures. The following day, the stacking gel was

made up and layered onto the running gel. The comb for sample wells was inserted immediately after pouring the stacking gel, which was allowed to set for 1 hour. The comb was then removed and the wells filled with running buffer. EBs were solubilized by boiling in the solubilizing buffer (0.1 M Tris-HCl pH 6.8, 2.5% (w/v) SDS, 0.5% 2-Mercaptoethanol, 20% glycerol and 0.01% Bromophenol Blue) for 10 minutes. 2-5 ug of protein was loaded into each well after solubilization and the gels were run in a Tris-glycine buffer at 25mA per gel until the tracking dye reached the bottom of the gel.

Molecular weight standards (Bio-Rad) were loaded alongside the samples for estimation of the molecular weights of resolved protein bands. The separated protein bands in the gel were either silver stained for visualization using the method of Morrissay (1981) or electro-transferred by a transblot apparatus (Bio-Rad) onto nitrocellulose membranes (NCM) by the method of Towbin et al. (1979). The transfer was usually made overnight using 0.15 amps followed by an increase to 0.22 amps for 1 hour the following morning.

The NCM with protein bands were cut into strips and blocked with either 4% BSA or 10% horse serum in Tris buffer (0.01 M Tris/0.15 M NaCl, pH 8.2) for 1 hour at 37^{o}C with shaking. The antibody to be tested was either used undiluted or diluted 1:10 in wash buffer (Tris buffer with 0.2% Tween 20 and 0.1% BSA). The NCM strips were immersed into 5-10 ml of antibody and incubated at

37°C for 1 hour. The strips were then washed three times in wash buffer and incubated with horseradish peroxidase conjugated anti-mouse immunoglobulins diluted 1:1500 in wash buffer at 37°C for 1 hour. The blots were washed again and developed with diaminobenzidine (Sigma, St. Louis, MO) at 0.5 mg/ml in PBS, pH 7.2 and hydrogen peroxide.

From the above screening methods, wells of interest were mini-cloned by removing the clones cells and replating them at 500-700 cells per 96-well microtitre plate using 20 ml of complete RPMI with HT (same as HAT except aminopterin was omitted) containing 5 x 10^7 thymocytes. The plates were fed complete RPMI + HT every 4 days until the cells were 50% confluent. The culture supernatants were tested as previously described. Positive wells were further subcloned at 30-50 cells per 96-well microtitre plate. At a ratio of 1 cloned cell per 3 wells, hybridomas that were monoclonal were obtained. From the 96-well plates, the clones were amplified into 24 well plates and then into 25 cm^2 flasks and 80 cm^2 flasks. They were also gradually weaned off thymocytes and HT medium as they were amplified.

3. Purification of IgG

Antibody to chlamydial antigens secreted by those hybridomas can be purified from either the hybridoma culture supernatants or from ascites fluid.

Ascites fluid was made by first priming the peritoneal cavity of 6-8 week old Balb/c female mice with 0.5 ml of pristine (Aldrich) or 0.5 ml of Freund's incomplete adjuvant (Difco). The mice were then rested for 1-7 days before being injected with 1-5 x 10^6 cloned cells intraperitoneally. Within 8-14 days, the peritoneal cavity of the mice would be swollen with tumor growth and ascites fluid would accumulate. The fluid, which contained antibody specified by the clone injected, was drained by inserting an 18-gauge needle into the peritoneum. The cell debris in the ascites fluid was removed by centrifugation at 10,000 g for 20 minutes. The cleared fluid was diluted 1:10 with Tris buffer, pH 8.0 before purification. Culture supernatants should be dialyzed against the starting buffer (0.05 M Tris, 0.15 M NaCl., 0.02% Na azide, pH 8.0) before purification.

IgG from culture supernatants or ascites fluid were purified by the use of a Protein-A sepharose column (Pharmacia). The Protein-A sepharose was reconstituted by swelling 5 g of powder in starting buffer. The slurry was poured into a 1.5 cm x 5.0 cm Econo Column (Bio-Rad) and allowed to settle until a steady flow rate was established. The packed column was then washed with another 50 ml of starting buffer before the sample was layered on top. Approximately 200 ml of culture supernatant or 20 ml of diluted ascites fluid could be processed per run. After the sample had run through, the column was again washed with 50 ml of starting

buffer to wash any unbound protein until the absorbance at 280 nm was zero. The bound antibody was eluted with 0.1 M acetic acid, 0.15 M NaCl, pH 4. One ml fractions were collected into 1 ml of 0.5 M phosphate buffer, pH 8.0 to neutralize the acidity of the eluting buffer and therefore prevent precipitation. The absorbance at 280 nm of each fraction was determined on a spectrophotometer. Fractions containing antibody were pooled and dialyzed against PBS. The column was washed with starting buffer and stored at $4^{o}C$.

E. Characterization of Mabs:

The antigenic specificity of the Mabs obtained from the fusions were characterized by different methods. In the dot-blot method, Mabs were tested against purified native EBs immobilized onto nitrocellulose membranes. This allows detection of surface exposed and conformational epitopes on native EBs. In the ELISA method, Mabs were reacted with EBs resuspended in a carbonate-bicarbonate buffer pH 9.6 and immobilized onto plastic microtitre plates. While the ELISA was used for screening because of convenience in doing large numbers of plates, the alkaline pH used in ELISA is known to change the EBs (Narita et al, 1976).In micro-immunofluorescence, the EBs were treated with 0.02% formaldehyde in PBS and acetone fixed onto glass slides. Microimmunofluorescence has been the traditional way of serotyping for C. trachomatis. It is

quantitative and is useful for knowing whether the Mab detected genus, species or type specific epitopes.

While in all tests described above, antibodies were reacted with whole EBs, in the immunoblot, antibody specificity to different proteins of chlamydia can be examined. In the immunoblot method, EBs were solubilized in a reducing detergent buffer and boiled for ten minutes. Thus the proteins in SDS-PAGE were denatured. However, they may be partially refolded when transferred onto NCM. It is therefore an important characterization assay as well. The ELISA and immunoblot procedures had been described in the previous section relating to monoclonal antibody screening and will not be repeated here.

1) The Micro-immunofluorescence Test (Micro-IF)

This test was designed by Dr. S.P. Wang, University of Washington, Seattle in 1971 for chlamydial serotyping and serology (Wang and Grayston, 1982) replacing the much more expensive and tedious mouse toxicity prevention test (MTPT) for serotyping isolates of C. trachomatis. It has become the gold standard in chlamyydial serology. Recently, serotyping of the chlamydial isolate by Mabs using Micro-IF has also replaced the traditional method of serotyping the sera from mice injected with the isolate.

The test consists of dotting a template of purified formalized EBs from each serovar onto a glass slide. 9 or 16 such templates could be made on each slide using

Hunt's finest pen nibs #104. After air drying, the slides were fixed in acetone for 15 minutes. Antibodies were put onto the template using a micropipette or a bacteriological loop. The slides were incubated for 30 minutes at 37°C and then washed three times in PBS and four times in distilled water. The fluorescein conjugated anti-mouse or anti-human second antibody was put onto the templates and again incubated at 37°C for 30 minutes. The slides were then washed as above, mounted in FA mounting fluid (Difco) with a coverslip and read in a Leitz fluorescent microscope.

2) The dot-blot assay

The dot-blot procedure was adapted from the method of Zhang et al, (1987). NCM was pre-soaked in PBS for 30 minutes before being mounted into the dot-blot apparatus. EBs (5 ug protein in 50 ul of PBS per well) were put into wells and allowed to filter through for 40 minutes. To minimize non-specific reactions on NCM, with 200 ul of 4% globulin-free bovine serum albumin (Sigma, St. Louis, MO) in PBS for 1 hour and then with 200 ul of PBS containing 0.2% Tween-20. Mab IgG (100 ul) was then put into each well and allowed to react for 1 hour at room temperature. The wells were washed three times with PBS-Tween 20 before adding 50 ul of ^{125}I-Protein A (Sp. act. 0.10 mCi in 0.205 ml ICN Radiochemicals, Irvine CA, 1 x 10^6 cpm/ml) per well. After 30 minutes, the NCM was washed again with three 200 ul aliquots of PBS-tween 20. The NCM was taken out

of the dot-blot apparatus and allowed to dry before being put into a Kodak cassette with x-ray film. The film was developed 4 hours later.

3) Isotyping of IgG

The purified IgG samples were isotyped by a dot-blot method developed in our laboratory by Ian Maclean. Rabbit antisera against mouse IgG isotypes 1, 2a, 2b and 3 (Miles Laboratories, Rexdale, ON) were diluted 1:5 in Tris buffer, pH 8.2. Fifty ul of each isotype antibody was allowed to bind to nitrocellulose membranes in a dot-blot apparatus (Bio-Rad) at room temperature for 30 minutes. The NCM was blocked with 4% BSA in Tris buffer at 37°C for 1 hour and then removed. One ml of a 1:50 dilution of Mab in Tris buffer with 0.2% Tween 20 and 0.1% BSA was then added and incubated for 1 hour at 37°C. The NCM was washed with washing buffer as in ELISA and incubated with HRP conjugated rabbit anti-mouse IgG at 1:3000 dilution for another hour at 37°C. The NCM was developed as for immunoblotting. The Mabs to be tested were compared to positive and negative controls included in the dot-blot.

4. Neutralization of infectivity by monoclonal antibodies

1. In vitro assays using coverslip cultures

The basic neutralization assay used in our previous work (Peeling et al., 1984) was varied to determine the effects of:

i) Antigen diluent

ii) Antibody Diluent

iii) Antigen concentration

iv) Antibody concentration

v) Temperature and duration of antigent-antibody incubation

vi) Volume of antigen-antibody reaction

vii) Cell cycle status of host cells

All solutions were adjusted to neutral pH and physiological salt concentrations and all experiments were performed in triplicate. Purified chlamydial EBs were diluted in SPG to give between 10^3-10^4 IFU per coverslip of Hela 229 cells. For all neutralization assays, the basic reaction, unless otherwise stated, was to combine 180 ul of the antigen with 180 ul of antibody and incubate the mixture at 37°C for 30 minutes with continuous shaking. The residual infectivity in the mixture was determined by inoculation onto 24 hour old coverslip cultures of HeLa 229 cells. Inclusions were stained and quantitated after 48-72 hours at 35-37°C.

The effect of using SPG or PBS as antigen diluent in the neutralization reaction was determined by using pure L_2 EBs diluted in SPG or PBS to give between 10^3 to 10^4 IFU per coverslip. The IgG concentrations of the neutralizating Mab, C11, was adjusted to a protein concentration of 80 ug/ml and three ten-fold dilutions were made in PBS containing 10 ug/ml of normal mouse IgG. These antigen and antibody preparations were

incubated at 37°C for 30' and residual infectivity quantitated in HeLa cell coverslip cultures.

As for the antibody diluent, it was found from previous experience that EBs are heat labile except in the presence of protein such as BSA or IgG. Thus to prevent excessive loss of viability of the inoculum, normal mouse IgG concentration was titrated against a constant concentration of EBs suspended in SPG and incubated for 30' at 37°C to find the minimal protective IgG concentration in the antibody diluent.

Having established a "protective" concentration for normal mouse IgG, the loss of EB viability in the presence of normal mouse IgG was compared with BSA at the same concentration over 60 minutes.

To determine the optimal antigen and antibody concentrations for the neutralization reaction, a checkerboard titration was performed. Pure L_2 EBs were diluted in SPG to give 10^5 IFU per coverslip from which three two-fold dilutions were made. The IgG from Mab UM-4 was adjusted to a protein concentration of 80 ug/ml and four four-fold dilutions were made in PBS containing 10 ug/ml of normal mouse IgG.

The temperature of incubation which would yield the best neutralization was determined as follows. Duplicate neutralization assays were performed using serovar L_2 EBs vs 4 concentrations of Mab UM-4 IgG. One set of the reaction mixture was incubated at 37°C for 30 minutes while the other set was incubated at 4°C for 60

minutes. Both sets were gently agitated on a continuous shaker.

To assess the effect of total volume of the reaction mix on neutralization, duplicate assays were performed according to the basic method using L_2 vs Mab UM-4. Just prior to incubation, the volume of 1 set of reaction mixture was brought up from 360 ul to 1 ml using 1:1 mixture of SPG and PBS containing 10 ug/ml of normal mouse IgG. The reactions were then incubated at 37°C for 30 minutes and residual infectivity was assayed on HeLa 229 cells.

Neutralization may also be affected by host cell cycle status. To determine this effect, Hela 229 cells were synchronized by applying a double thymidine block over two 16 hour periods with an 8 hour release interval using 2 mM thymidine in growth media. The neutralization of infectivity of serovar L_2 EBs by Mab UM-1 IgG was assayed on both synchronous and asynchronous cells.

After the optimal conditions for the neutralization assay was established, all Mabs produced were tested for neutralization of infectivity against various serovars.

2. Mechanism of neutralization

i) Attachment and endocytosis assays

EBs were intrinsically labelled with ^{14}C as previously described (Peeling et al. 1984). Briefly, EBs were inoculated onto HeLa 229 cells in 175 cm^2

flasks. The culture medium was replaced at 10-12 hours post-infection with MEM containing 10% dialyzed fetal calf serum, 5 uCi ^{14}C-amino acids/ml (Sp. Act. 1.68 mCi/mg, ICN Radiochemicals, Irvine, CA) and 1 ug/ml emetine or cycloheximide. At 48 hours post-infection, the monolayer was harvested and ^{14}C labelled EBs were purified by renografin gradient centrifugation.

The rate of attachment to and uptake of EBs in HeLa cells were measured by allowing radiolabelled EBs treated with Mab IgG or normal mouse IgG to attach to 24 hour old HeLa monolayers at 4^{o}C (for attachment) or room temperature (for endocytosis) for 15, 30, 60 and 120 minutes. At the end of each time interval, the inocula were removed by aspiration and the monolayers washed five times with cold phosphate-buffered saline (PBS). One half of the coverslips, were treated with heparin (100 u/ml in PBS) for 30 minutes to release attached but unendocytosed organisms. Coverslips were again extensively washed. Preliminary experiments indicated that heparin could release 80% or more of cell associated EBs of serovar L_2. The monolayers, with or without heparin treatment, were then solubilized in 2% SDS and counted in a liquid scintillation counter using 7.5 ml of Scintiverse II (Fischer Scientific, Fair Lawn, NJ) per sample. The number of attached EBs correspond to the total cell-associated counts per minute (cpm). The number of endocytosed EBs correspond to the heparin resistant cell-associated cpm.

ii) Fluorescent staining of intracellular EBs

To follow the intracellular fate of the neutralized EBs, serovar L_2 EBs were incubated with Mab, UM-4 or normal mouse IgG at 37°C for 30 minutes. The reaction mixtures were allowed to adsorb to HeLa 229 monolayer cultures at room temperature for 2 hours. Attached EBs which were not internalized were released with heparin and the monolayers then fixed at 2, 4, 8 and 20 hours post-infection. Half of these monolayers were then stained with the Syva Microtrak fluorescent antibody to C. trachomatis to monitor normal EB development and the other monolayers were stained with fluorescein-conjugated anti-mouse IgG to visualize the antibody-treated EBs.

3. In vivo assay: Mouse Toxicity Protection Tests

3-4 week old Balb/c mice were injected intravenously with varying doses of pure EBs (10^6-10^9IFU/ml) to establish the lethal concentration to be used in subsequent protection tests. In the protection test, lethal doses of EBs were incubated with 200 ug/ml of UM-1 IgG at room temperature for 1 hour with constant shaking. Normal mouse IgG at the same concentration was used as control. These reaction mixtures were then injected intravenously into mice and deaths within 24 hours were recorded. Six mice were used per dose, and heat inactivated EBs at the lethal concentration were used as controls.

F. Trypsinization studies

1) Trypsinization of EBs and infectivity assays

Purified whole EBs (50 ug protein) were incubated with trypsin in Tris-saline buffer, pH 7.8, at final concentrations of 1 mg/ml, 100 ug/ml or 10 ug/ml at 37°C. For infectivity assays, EB samples were taken after 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours and 8 hours of trypsinization. Control EBs were incubated with Tris-saline buffer alone and were collected at the same time intervals. The protease inhibitor, phenylmethanesulfonyl fluoride (PMSF, Sigma Chemical Co., St. Louis, MO), known not to inhibit chlamydial infectivity (Hackstadt and Caldwell, 1985), was added to a final concentration of 2 mM to both control and trypsinized samples at the end of each incubation period to stop further proteolysis. Aliquots of EBs were also used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The morphology and state of aggregation of control and trypsinized EBs were examined in electron microscopy by negative staining using 2% phosphotungstic acid.

2) Attachment and endocytosis assays

^{14}C labelled EBs (serovar L_2) were trypsinized at 37°C for 30 min. PMSF was added to 2 mM to stop the reaction. The EBs were then used in attachment and endocytosis assays as previously described [Section E.2.].

3. ^{125}I-lactoperoxidase labelling

Purified EBs were treated with 1 mg/ml trypsin or buffer alone for 15 minutes. The reaction was stopped by adding PMSF to a final concentration of 2 mM. EBs were pelleted in a microcentrifuge and resuspended in 500 ul PBS. Iodobeads (Pierce Chemicals Co., Rockford, Ill.), previously equilibrated for 5 minutes in PBS, were put into each microcentrifuge tube and 0.33 uCi ^{125}I (ICN radiochemicals, Irvine, CA) was added. The reaction was allowed to proceed for 15 minutes with occasional shaking. Iodobeads were removed and the reaction mixture spun in a microcentrifuge for 15 minutes. The pellet, containing radio-iodinated EBs, was resuspended in 300 ul of solubilizing buffer, boiled for 10 minutes and then run on 12% SDS-PAGE. The resolved protein bands were electrotransferred onto NCM. The immunoblot was developed using various Mabs and then put into a film cassette with Kodak T-mat G x-ray film and developed after 12 hours.

G. Biochemical Assays

1) RNA Polymerase Activity Assays

Purified EBs (500 ug protein) were treated with 1 mg/ml of trypsin in Tris-saline buffer, pH 7.5 for 30 minutes at 37^{o}C. Control EBs were incubated with Tris-saline buffer pH 7.5. The EBs were then pelleted at 30,000 g for 30 minutes at 4^{o}C and resuspended in 100 ul of transcription buffer containing 0.5 mM Tris-HCl,

pH 7.5, 2 mM $MgCl_2$ and 300 mM KCl. The EBs were then added to an equal volume of transcription reaction mix containing 0.5 mM each of cytosine-triphosphate (CTP), guanosine triphosphate (GTP), adenosine triphosphate (ATP) and 10 uM of ^{32}P-labelled uridine triphosphate (^{32}P-UTP) (Sp. Act. 752 Ci/m mole, ICN Biochemicals, Irvine, CA). Controls were prepared without the unlabelled nucleotide triphosphates. At 0 time, a 4 ul aliquot was removed to assay for total count in the sample. The reactions were allowed to proceed at 25^{o}C. At 15, 30, and 60 minutes, 40 ul aliquots were taken out and put into 1 ml of ice cold 10% trichloroacetate acid containing 1% (w/v) sodium pyrophosphate (TCA-p). Samples were kept on ice for at least 10 minutes before being filtered on glass fibre GF/B filters. The filters were washed with 30 ml of TCA-p, rinsed in absolute alcohol and dried before being placed in 5 ml. POPOP-toluene for scintillation counting.

EBs were also treated with 2-mercaptoethanol as described by Sarov and Becker (1971) and assayed for RNA polymerase activity. 2-5 x 10^7 EBs which were first frozen in a buffer containing 0.1 M Tris-HCl pH 7.5, 8% sucrose, 1 mM 2-ME and 1 mM $MgCl_2$. On thawing, they were resuspended in transcription buffer containing 2 mM 2-ME to give a final concentration of 1 mM 2-ME when combined with an equal volume of transcription reaction mix. The assay was then performed as described in the preceeding section.

2. High Resolution ^{31}P Nuclear Magnetic Resonance Spectroscopy (NMR): Assay for ATPase activity

Purified EBs were filtered through a 0.45 um filter to prevent RB contamination in the sample. EBs were then treated with 1 mg/ml trypsin or 2 mM 2-ME at 37°C for 30 minutes, pelleted in a microfuge, and resuspended in ATP buffer containing 10 mM Tris, HCl, pH 7.5, 2.2 mM $MgCl_2$, 5mM KCl 100 mM NaCl, 50 uM Na_2EDTA and 2 mM ATP. 10% D_2O was added to provide a lock signal for the spectrometer. ^{31}P-NMR spectra were obtained at 121 MHz (7.1 Tesla) using a Bruker AM-300 spectrometer (Bruker Spectrospin, Karlsruke, Germany) with a sample temperature of 310 K. For each sample 400 FIDS (free induction decay) were accumulated into 15 K data points using a spectral width of 4630 Hz (digital resolution 0.565 Hz/data point) and an acquisition time of 1 second with an inter-pulse delay of 2.5 sec. A line broadening of 2 Hz was used prior to Fourier transformation to give the NMR spectrum. Measurements of ATP and inorganic phosphate (P_i) arising from the hydrolysis of ATP, were made for each sample. Chemical shifts were referenced to external 75% H_3PO_4 (0 ppm). ATPase activity was calculated from the ratio of intensities of the Pi peak to that of the γ - phosphate of ATP.

3) Inhibition studies using neutralizing Monoclonal Antibodies

In experiments to determine if neutralizing MOMP Mab UM-4 inhibit RNA polymerase activity, 288 ug of

control or Mab IgG were incubated with 3 concentrations (10^7, 5 x 10^7 and 10^8) of purified L_2 EBs for 30 minutes at 37°C before 2-ME was added to a concentration of 2 mM in the neutralization reaction. This was then incubated for a further 30 minutes at 37°C before combining with the transcription reaction mix. The transcription reaction was allowed to proceed for 45 minutes at 25°C. TCA precipitated ^{32}P-UTP incorporated was processed as previously described.

To assess if the presence of 2 mM 2-ME affected the antigen or the antibody in the neutralization reaction mix, EBs were treated with 2 mM 2-ME or Tris buffered saline alone for 30 minutes at 37°C prior to combination with antibody. They incubated at 37°C for a further 30 minutes. Residual infectivity from these two treatments were compared with that of the basic neutralization reaction.

In experiments to determine if neutralizing MOMP Mabs can inhibit ATPase activity, 10^8 EBs were first incubated with 288 ug/ml of IgG from normal mouse serum, an anti-LPS Mab or neutralizing Mab to MOMP at 37°C for 30 minutes before 2 ME was added to 2 mM concentration in the reaction mixture. This was then combined with an equal volume of ATP buffer containing 2 mM ATP and measurements of P_i made as described.

RESULTS

A. Life cycle of C. trachomatis by transmission and scanning electron microscopy

1. Attachment and Uptake

From scanning electron micrographs Chlamydia trachomatis serovar J appeared to make its initial contact, mainly though not exclusively, with the microvilli of the HeLa 229 cell surface (Figure 1a). This was also evident in transmission electron micrographs of thin sections through an infected cell monolayer (Figure 1b), which showed that the contact point was the tip of microvilli rather than the cell surface. Figure 2a and 2b show that EBs did not appear to be internalized through clathrin-coated pits. These pits are usually identified by a thick coating of dark staining bristle-like structures on the cell surface using the glutaraldehyde tannic acid saponin fixation method. (Fig. 2c) The average diameter of these pits is 100 nm. No actin-like filaments were evident around the area of the endosome.

2. Evasion of Phagolysosomal Fusion and Replication

Figure 3 shows an EB inside a membrane bound endosome four hours post-infection. There was no evidence of lysosomal fusion as the EB remained structurally intact. At eight hours post-infection, the EBs reorganized into RBs and started replicating by binary fission (Figure 4). During transition of EB to RB the dense nucleoid material in EBs had apparently

dispersed. The cytosol became more granular. The granular material appeared to be mainly ribosome-like structures associated with irregular areas of low electron density containing strands of fibrillar material. Replicating RBs appeared to marginate the inside of the inclusion membrane (Figure 11). The outer membrane of RBs often appeared fused in places to the cytosol side of the inclusion membrane. Many mitochondria and rough endoplasmic reticulum were seen around the inclusions. Replication is asynchronous such that by 36 hours, RBs of varying sizes and internal densities were apparent and some had condensed to EBs (Fig. 5). At this time, by SEM, many HeLa cells showed huge bulges as a result of expanding chlamydial inclusions (Figure 6).

3. Release of Mature EBs

The maturation of the chlamydial inclusion led to the release of the infectious EBs from the host cell. There appeared to be two main mechanisms of release. In the first mechanism, the inclusion appeared to be extruded intact from the host cell (Figure 7a and b). One can see the connection between the cell and the inclusion starting to tear (Fig. 7b) Hundreds of EBs can be seen enclosed inside the thin translucent inclusion membrane (Figure 8). Two outcomes may result. In some cases, the inclusion appeared to be extruded with such force that the host cell nucleus is extruded as well, presumably resulting in cell death. The

apparently gentler extrusion of the inclusion resulted in a deep cavity in the host cell (Fig. 10a and b). The host cell in Fig. 10b shows on its left side, the remnant of an inclusion meembrane. The cavity was still membrane lined so it might eventually survive by repairing itself. The second mechanism of release appeared to involve rupture of the host cell plasmalemma and mature release of EBs directly into the external medium outside (Figure 9). A possible consequence of this form of release can be seen in the thin section where residual EBs and RBs are marginated along the inclusion membrane remained. (Figure 11, 12a). Figure 11 clearly shows the remnants of an inclusion membrane (im). Part of the inclusion membrane appeared to have collapsed inward after the extrusion. EBs and RBs in all stages of development were left inside the inclusion. Some RBs were still fused to the inclusion membrane. Fig. 12a shows a thin section through an infected cell where the nucleus of the cell appeared relatively intact. The inclusion was empty but again there were EBs and RBs left inside. Fig. 10c shows the HeLa cell monolayer at 75 hours post-infection. The monolayer was still intact even though there were many cells left with large craters. By 92 hours post-infection, most of the cells had appeared to be in repair with a thin membrane growing over the crater where the inclusion has been. EBs can be seen inside of some of the cells undergoing repair.

FIGURE 1 Attachment of C. trachomatis EBs to HeLa cells

a Scanning electron micrograph of EBs on the surface of a HeLa cell

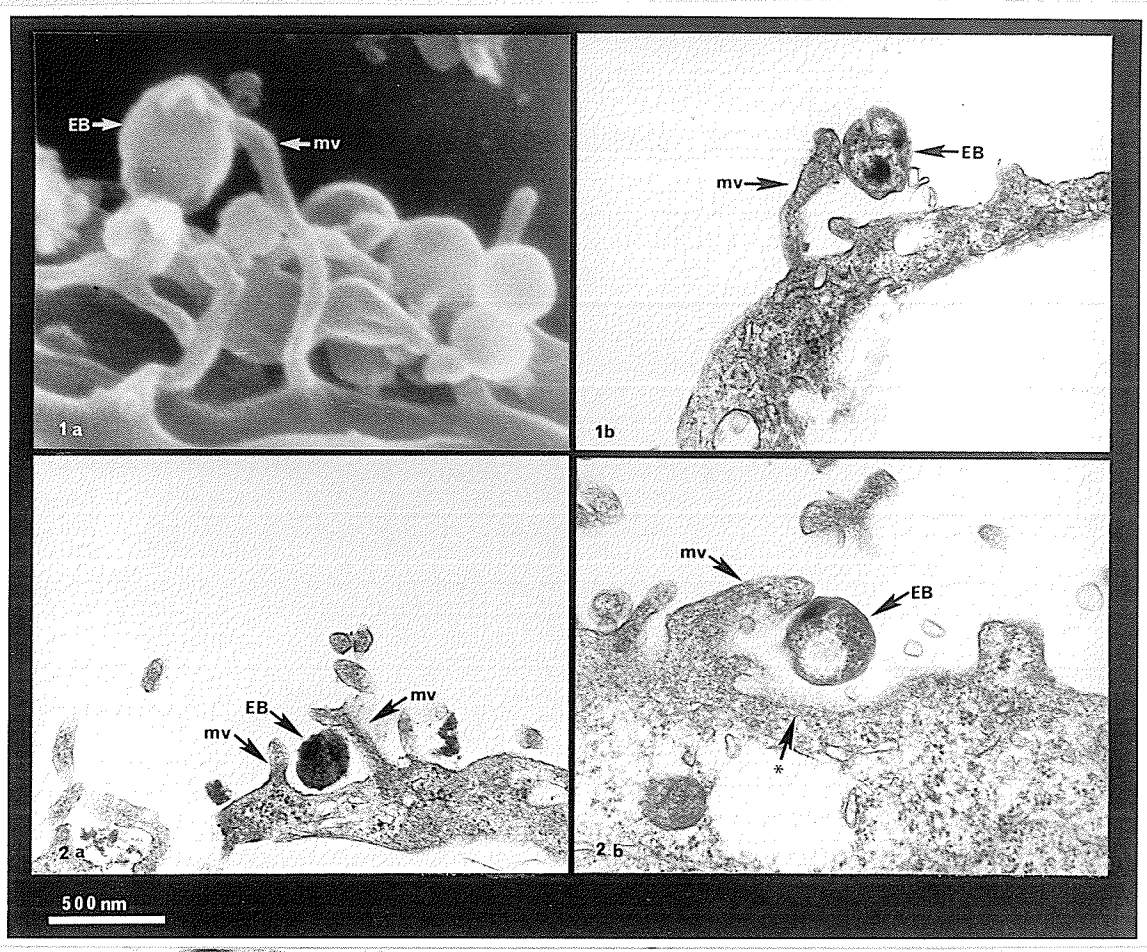
b Transmission electron micrograph of a thin section of a HeLa cell showing an EB in contact with the host cell microvillus

(EB = Elementary body; mv = microvillus)

FIGURE 2a and b Endocytosis of C. trachomatis EBs into HeLa cells

*Indicates the lack of clathrin-coated pit staining at the site of internalization

c Clathrin-coated pit on HeLa cell surface


EB
mv
1 a
mv
EB
1b
EB
mv
mv
2 a
mv
EB
*
2 b
500 nm

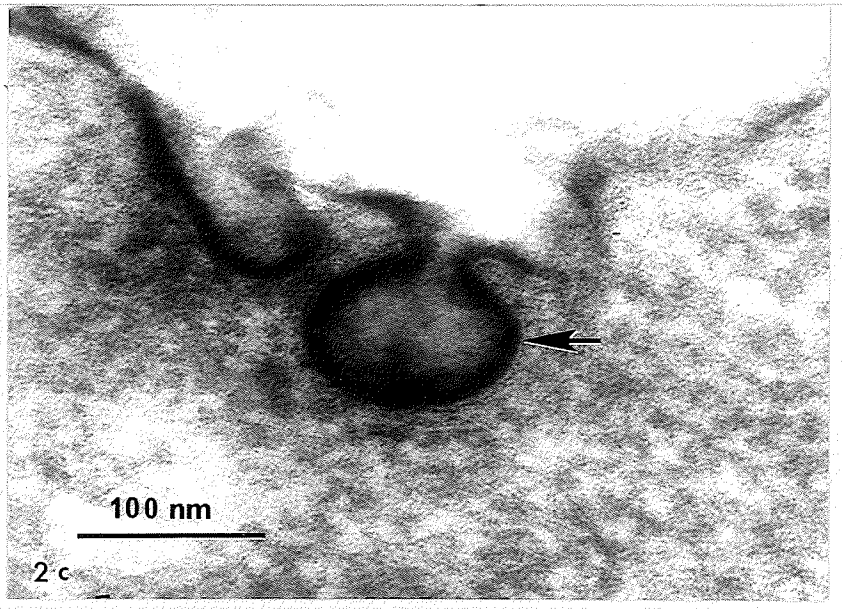
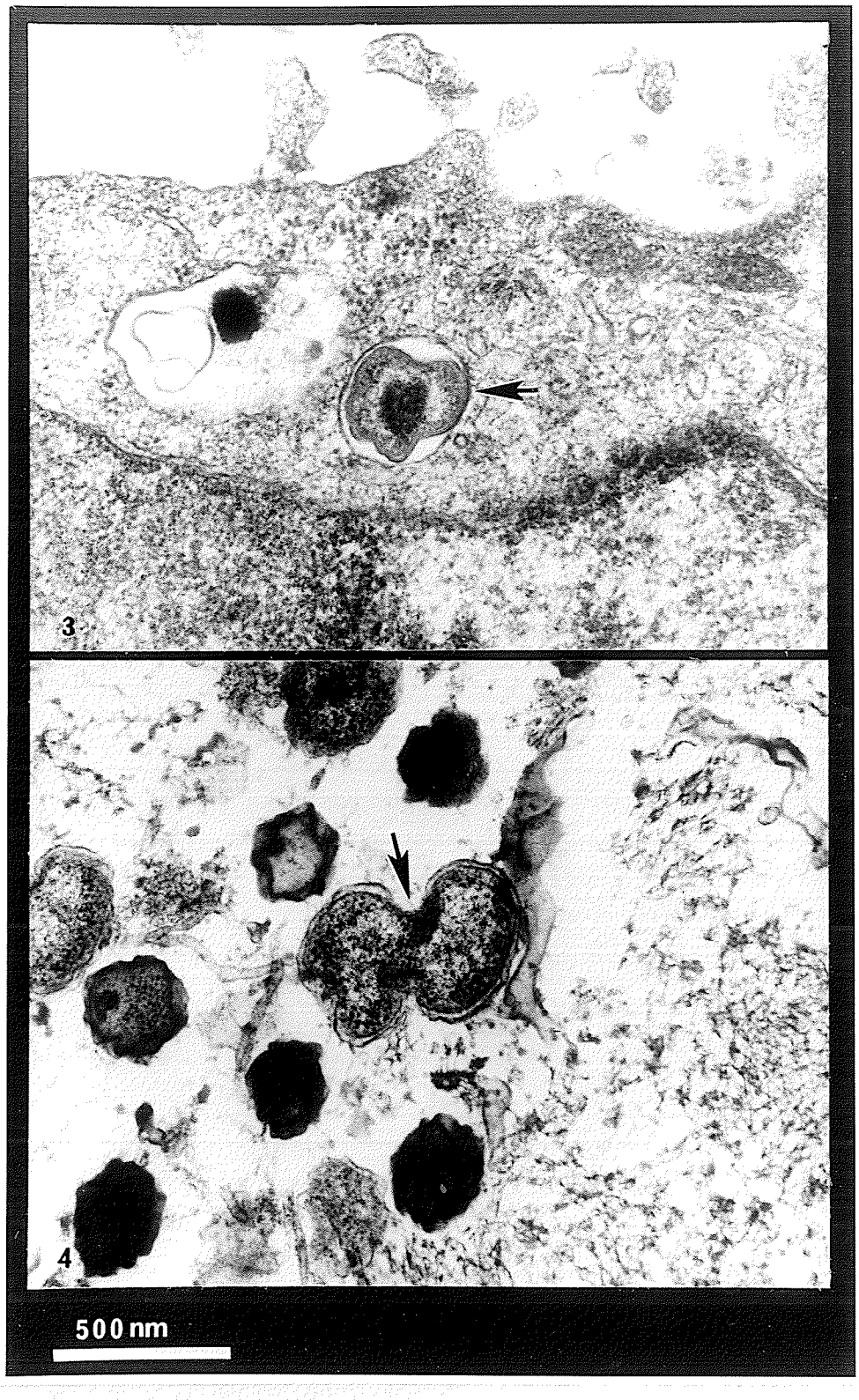

100 nm
2 c

FIGURE 3. C. trachomatis EB in an endosome 4 hours post-infection

FIGURE 4. C. trachomatis RB in the process of binary fission inside an inclusion


3
4
500 nm

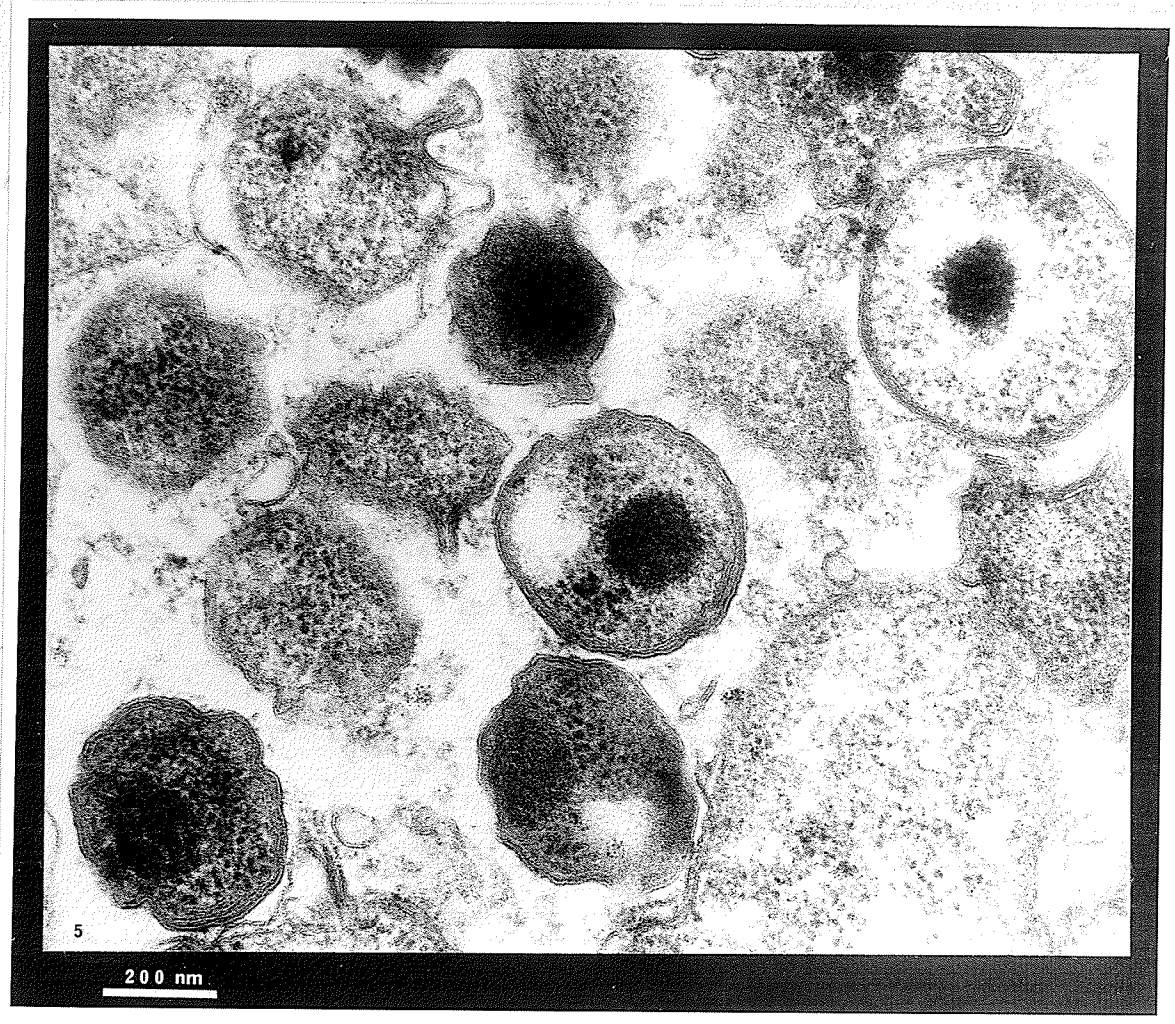


FIGURE 5. C. trachomatis EBs and RBs inside an inclusion 36 hours post-infection, showing the asynchronicity of replication

FIGURE 6a & b. Scanning electron micrograph showing a HeLa cell monolayer 36 hours post-infection. Many bulging cells (c) can be seen as a result of growing intracellular chlamydial inclusion

FIGURE 7a & b. Scanning electron micrographs showing the extrusion of chlamydial inclusions from HeLa cells 48 hours post-infection. (* = inclusion tearing away from host cell)

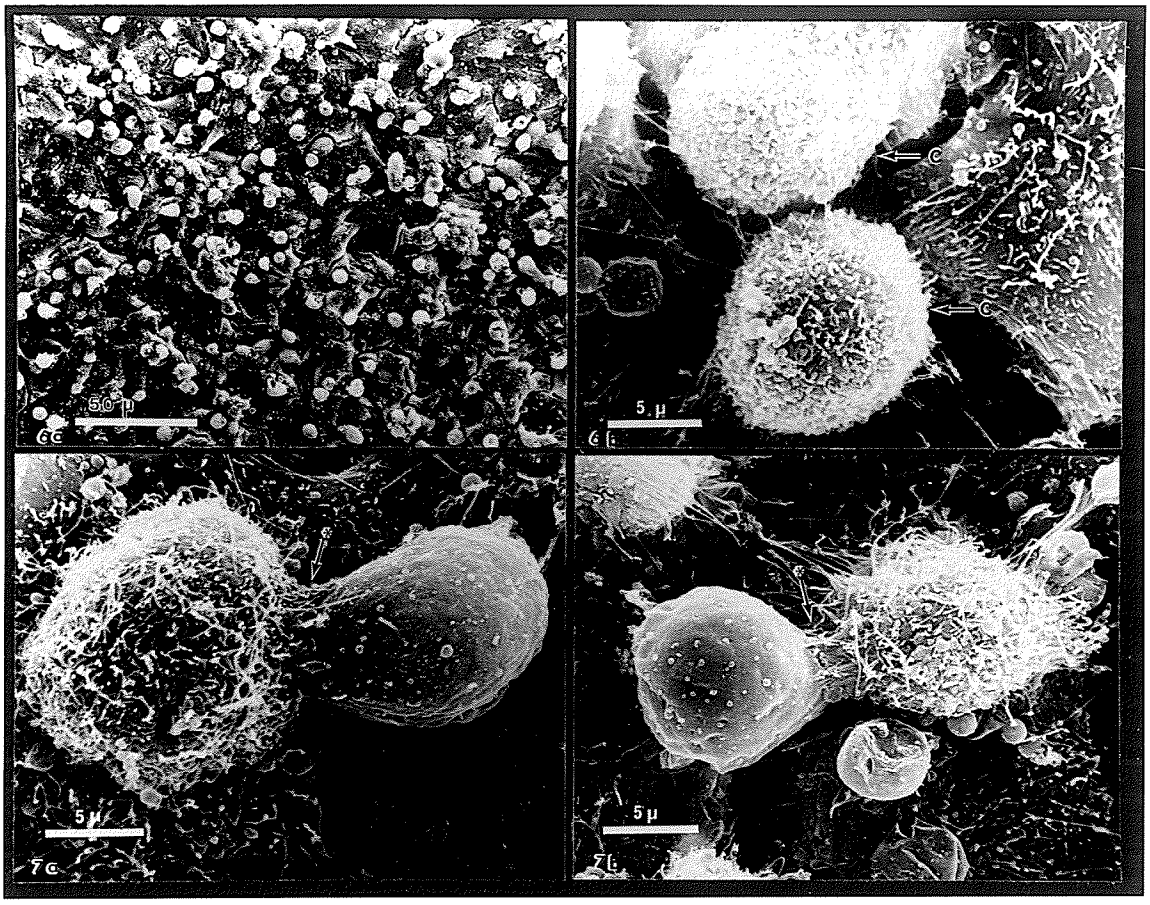
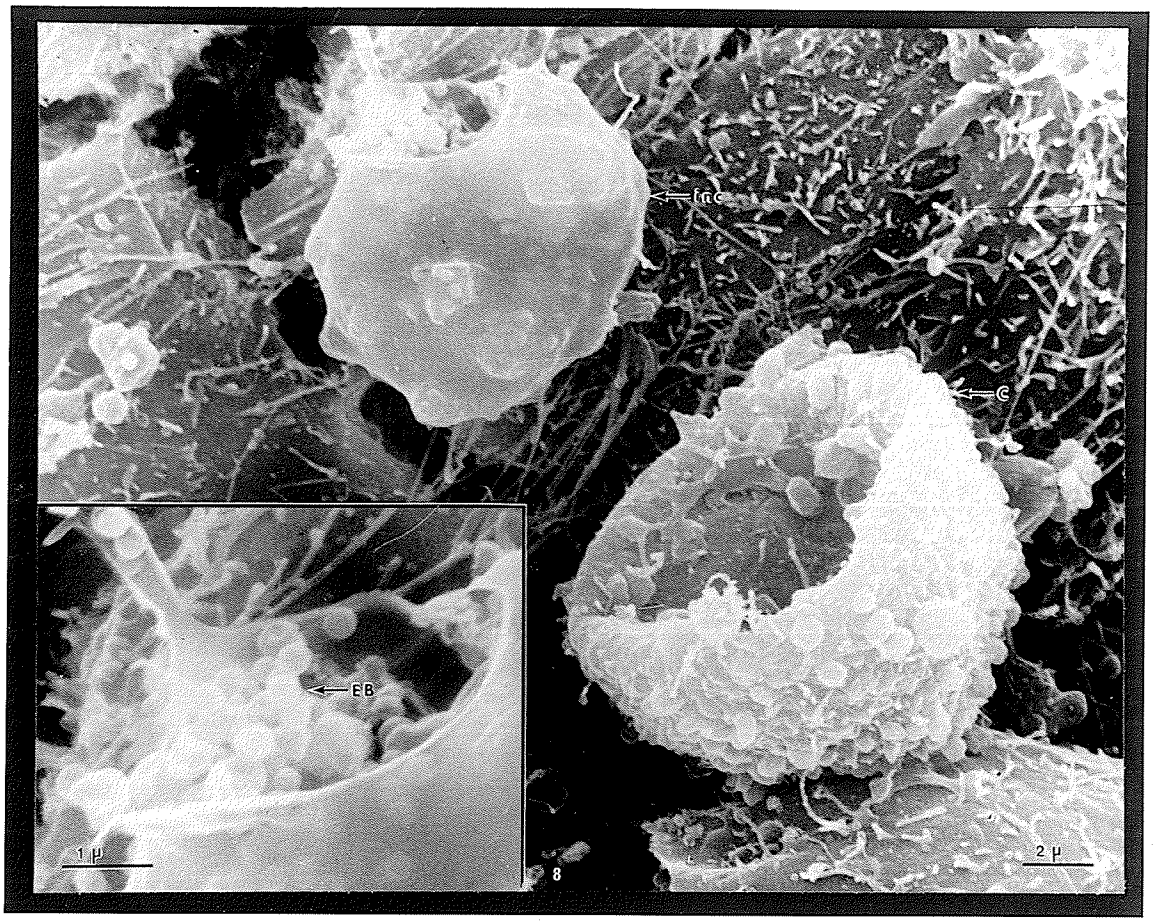

50 µ
6a
C
C
5 µ
6b
H
5 µ
7a
5 µ
7b

FIGURE 8. Release of mature chlamydial inclusion from a HeLa cell 48 hours post-infection (inc = inclusion; c = host cell)

FIGURE 9. Release of C. trachomatis EBs from an infected HeLa cell 48 hours post-infection (c = host cell)


fmc
C
EB
1 µ
2 µ
8

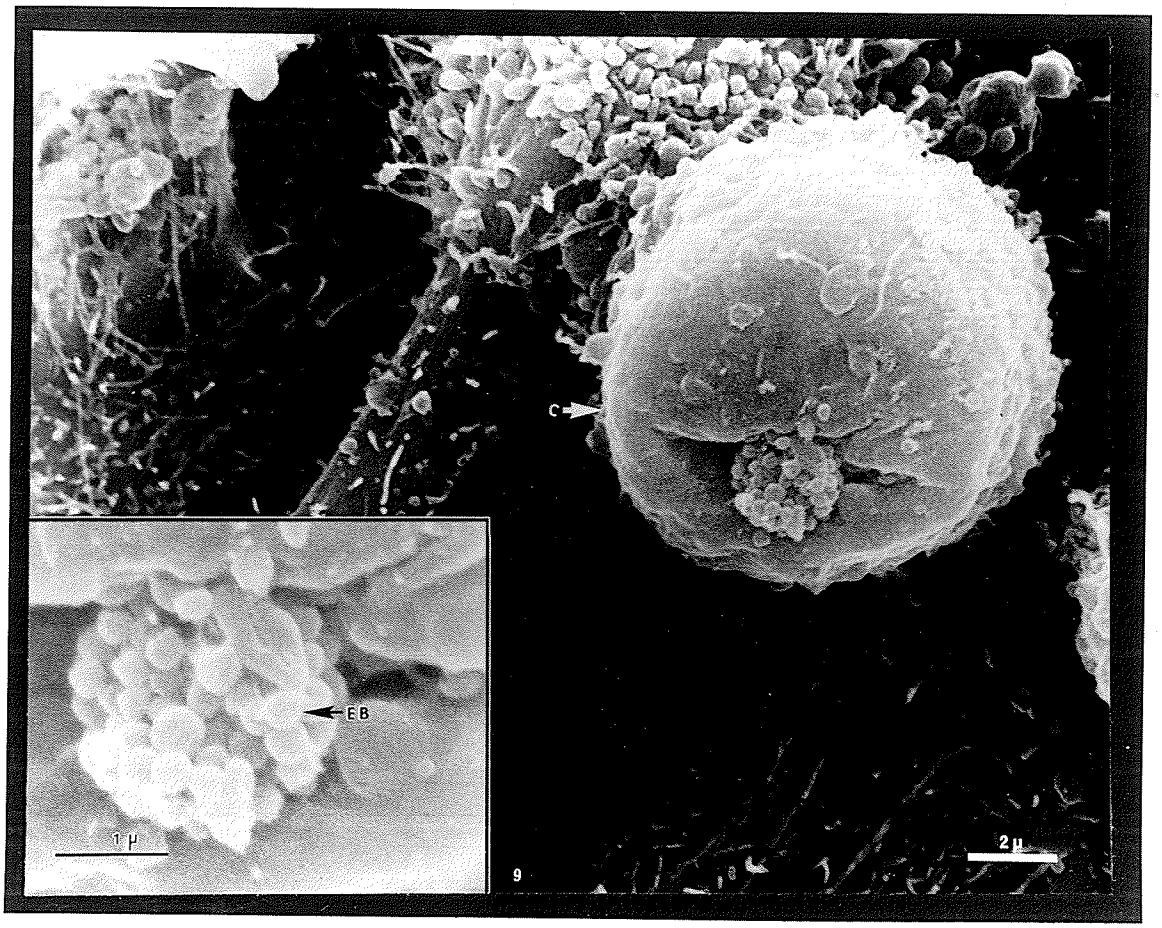

C
EB
1 µ
2 µ
9

FIGURE 10a,b. Infected HeLa cell after extrusion of chlamydial inclusions 48 hours post-infection

FIGURE 10c. Scanning electron micrographs of a HeLa cell monolayer at 75 hours post-infection with C. trachomatis. Arrows point to crater-like structures left after extrusion of chlamydial inclusions

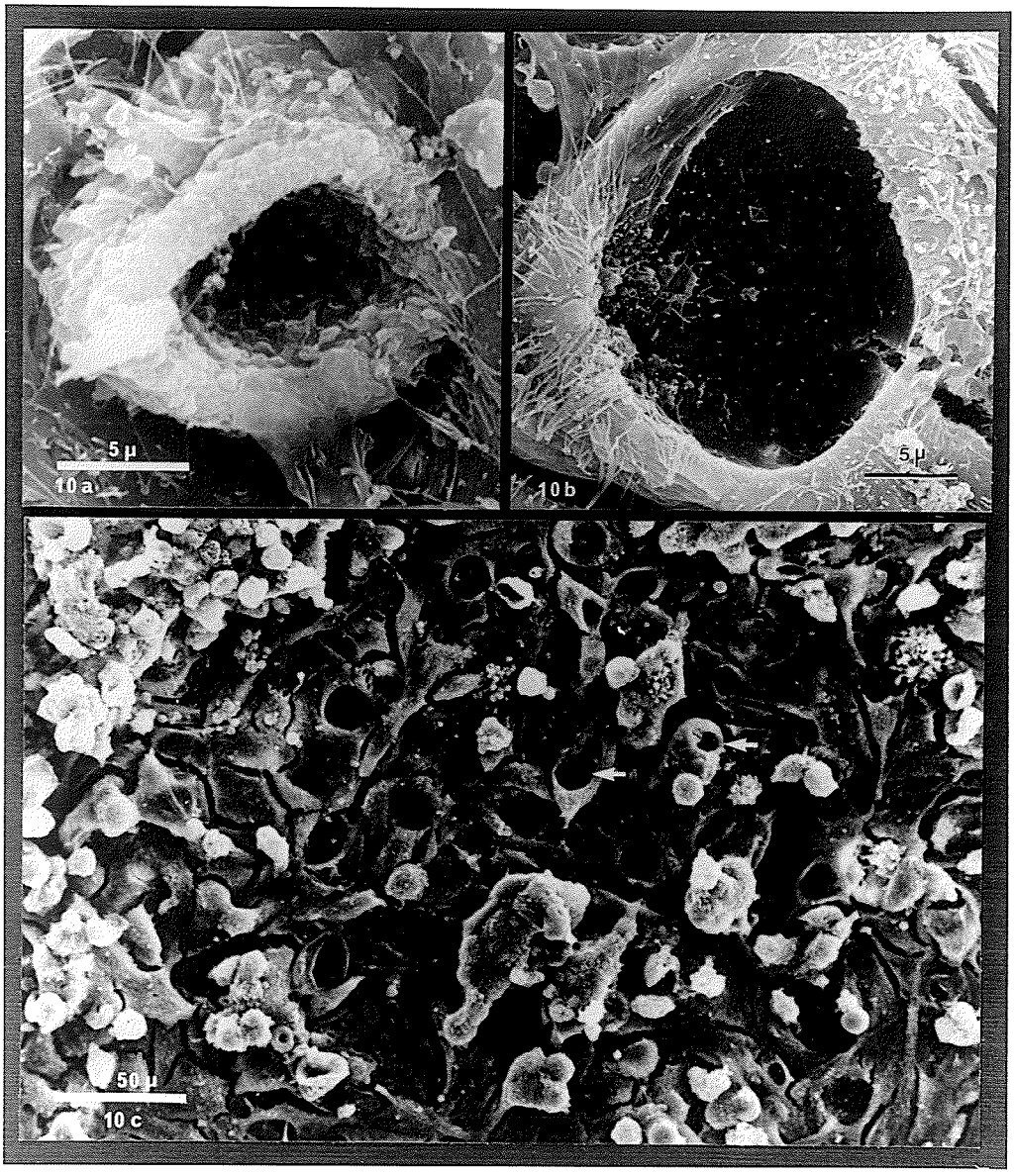

5 µ
10 a
10 b
5 µ
50 µ
10 c

FIGURE 11. Transmission electron micrograph showing a thin section of an infected HeLa cell 75 hours post-infection. EBs and RBs can still be seen inside the inclusion after most of its content has been released.

EB = Elementary body

RB = Reticulate body

im = inclusion membrane

mi = mitochondria

rer = rough endoplasmic reticulum

Arrow in inset points to apparent fusion of RB outer membrane to inclusion membrane

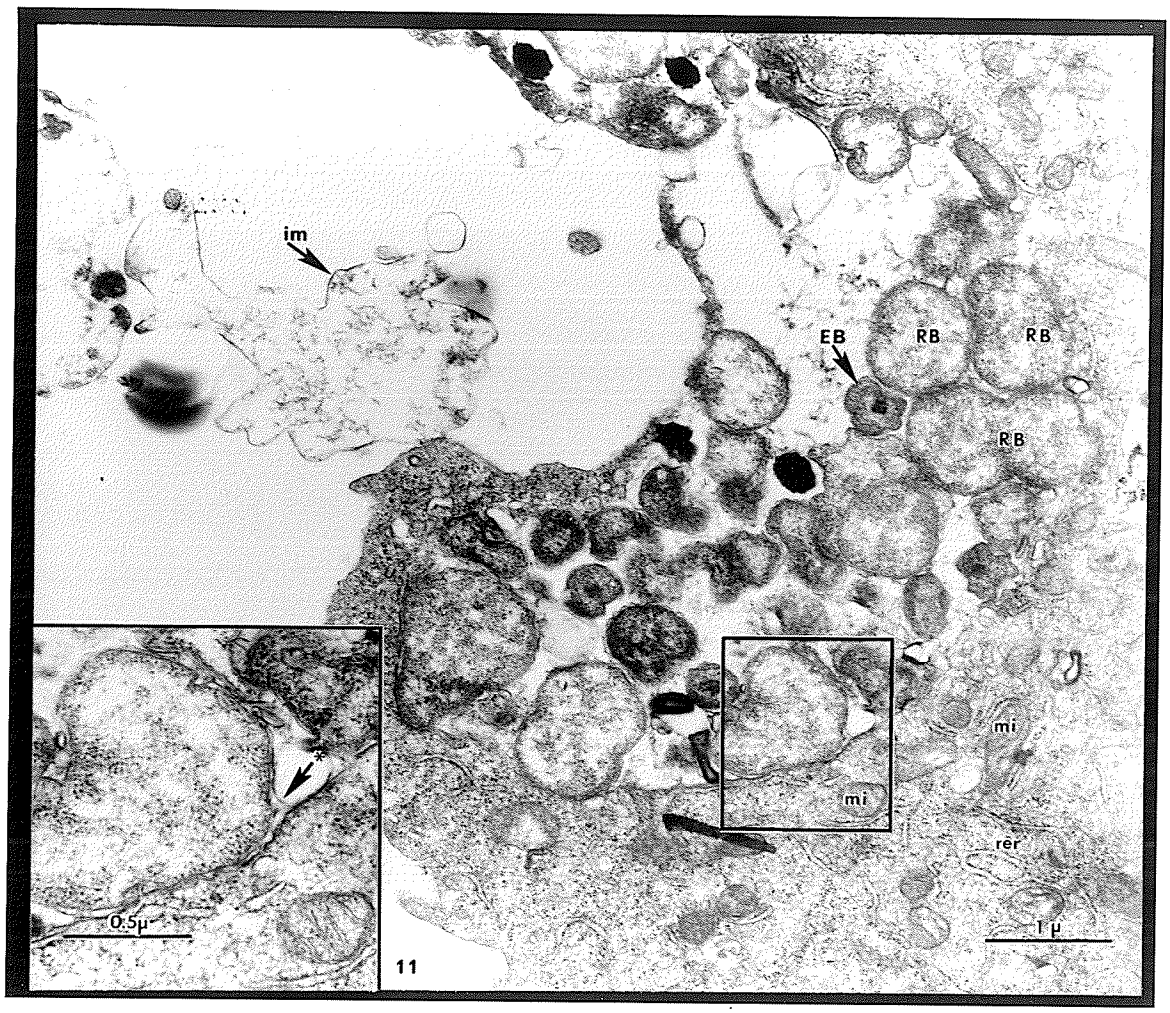
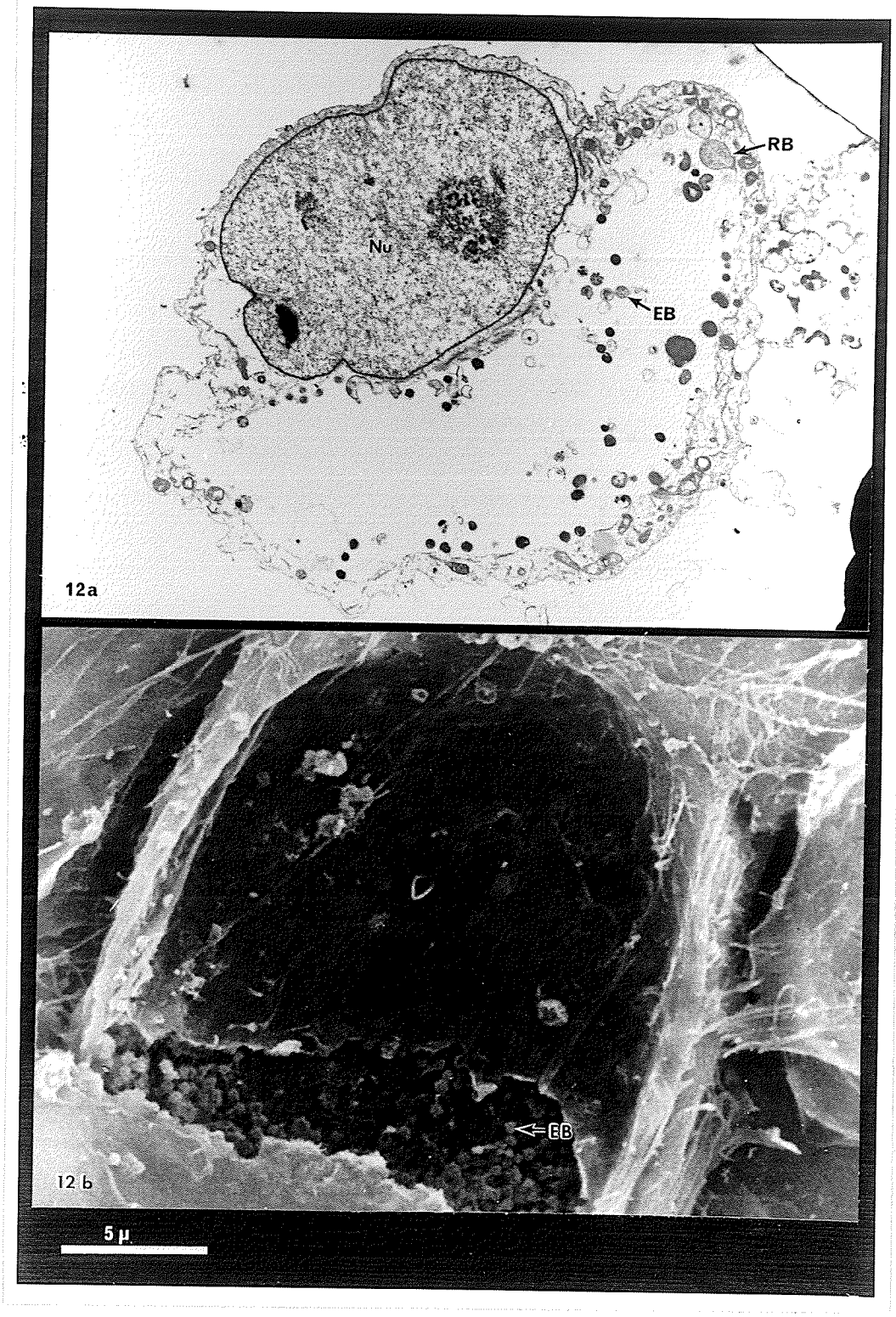

im
EB
RB
RB
RB
mi
mi
rer
0.5µ
1 µ
11

FIGURE 12. HeLa cells in repair 92 hours post-infection

a) Transmission electron micrograph of a thin section through an infected cell with intact nucleus (nu). Most of the contents of the chlamydial inclusion had been released but some EBs and RBs remained

b) Scanning electron micrograph of an infected HeLa cell where a thin membrane covered the inclusion crater. Residual EBs can be seen inside


RB
Nu
EB
12a
EB
12 b
5 µ

In summary, the interaction of serovar J with HeLa cells began with EBs contacting the microvilli of host cells and being endocytosed by a mechanism which did not involve clathrin-coated pits. Inside membrane bound vesicles, EBs differentiated into RBs. RBs were often seen on the periphery of inclusions with their outer membrane contiguous with the inclusion membrane. The inclusions were surrounded by host cell mitochondria and rough endoplasmic reticulum. RBs replicated by binary fission. The multiplication was asynchronous. RBs condensed into EBs. Mature EBs were released from the host cell either individually through a break in the plasmalemma or the inclusion was extruded as a whole. Some cells appeared to be in repair post-infection.

These ultrastructural studies define stages of the chlamydial life cycle which are amenable to study using antibody-mediated neutralization. These are the early events of attachment, endocytosis, inhibition of phagolysosomal fusion and EB to RB differentiation. Antibody to EB outer membrane antigens may inhibit any or all of these events.

B. Production and Characterization of Monoclonal Antibodies

1. Production of monoclonal antibodies

Figure 13 shows a silver-stained SDS-Polyacrylamide gel of solubilized EBs from 12 C. trachomatis serovars, the mouse pneumonitis strain of C. trachomatis and the C. psittaci meningopneumonitis strain. Over fifty

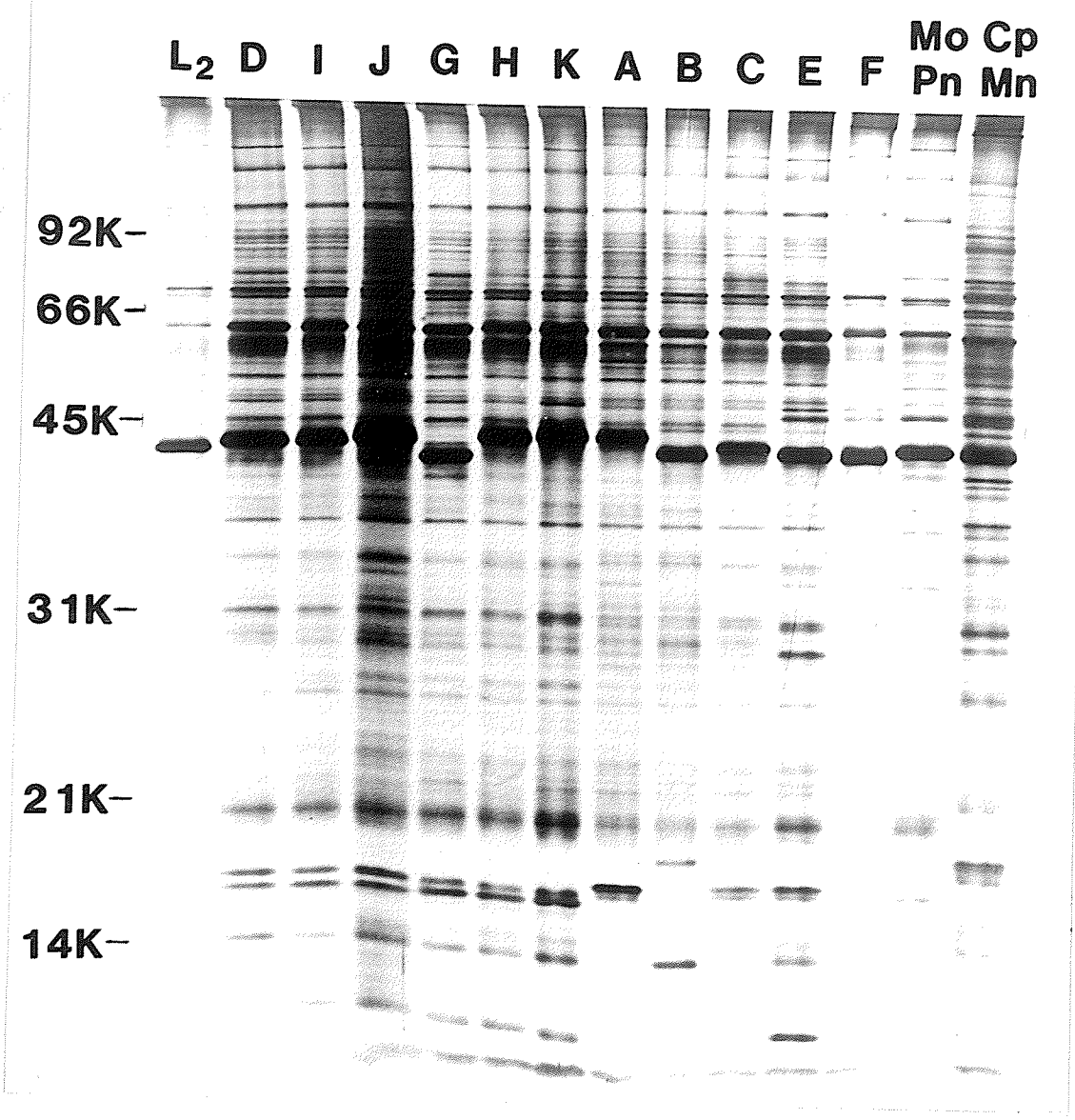


FIGURE 13. Silver-stained SDS-PAGE resolution of chlamydial macromolecules. Lanes representing serovars of C. trachomatis: A-K, L_2 and mouse pneumonitis (MoPn) and C. psittaci, meningopneumonitis strain (CpMn), are labelled across the top. Resolved macromolecules were calibrated by molecular weight markers as shown on the left.

different molecular species were separated according to molecular mass. The most prominent of these was the major outer membrane protein which is common to the genus with a variable molecular mass of between 39 kDa and 43 kDa.

Figure 14 is an immunoblot showing the reactivity of the mouse sera from the fusion with serovar G against all the chalmydial macromolecules resolved in SDS-PAGE. The mice recognized macromolecules of molecular mass 10 kDa, 29 kDa, 32 kDa, MOMP (40 kDa), 57 kDa, 60 kDa, 75 kDa and to a lesser extent a number of high molecular weight molecules.

Monoclonal antibodies were produced to each of these antigens. In all, 99 hybridomas were produced. We identified Mabs specifying epitopes on 8 different antigens from three independent fusions using serovar L_2, J, and G as immunogens. The culture supernatants of these clones were screened for neutralization of infectivity against the homologous immunizing serovar. From the various screening tests, 13 clones of interest were amplified and purified IgG produced from culture supernatant or ascites fluid. Additionally, one Mab, designated C11 was kindly provided to us by Dr. J. Mahoney of McMaster University, Hamilton, Ontario. Thus, 14 Mabs were studied in detail.

2. Characterization of Monoclonal Antibodies

Table 5 and 6 summarize the characterization of Mabs according to IgG isotype, macromolecular serovar

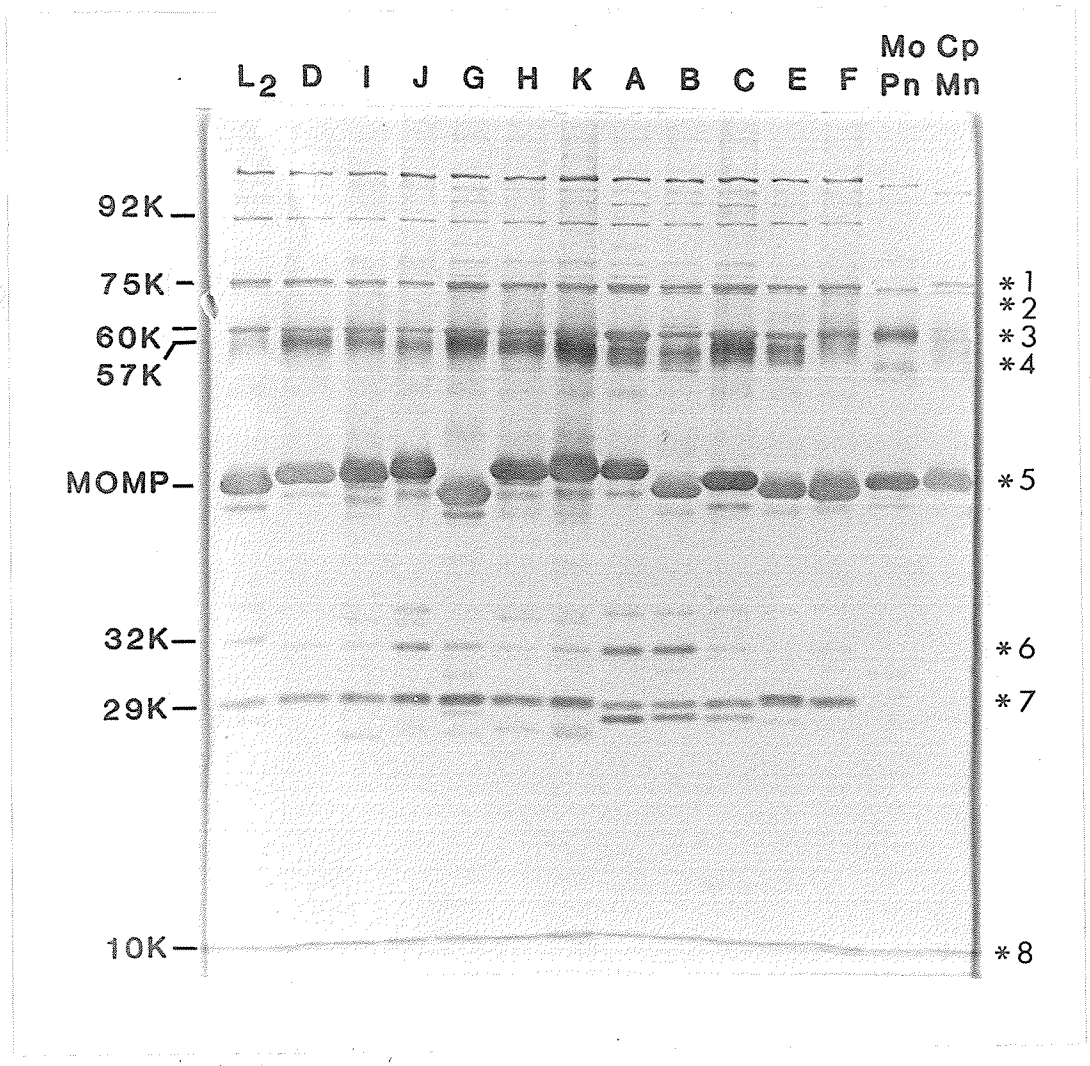


FIGURE 14. Immunoblot showing reactivity of mice sera from the fusion of serovar G against macromolecules of chlamydiae resolved by SDS-PAGE. Molecular weight calibrations are shown on the left side. Lanes representing C. trachomatis serovars A-K, L_2 and mouse pneumonitis (MoPn) and C. psittaci meningopneumonitis strain are labelled across the top.

* indicates antigens to which monoclonal antibodies have been produced

TABLE 5

CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO THE MAJOR OUTER MEMBRANE PROTEIN (MOMP) OF C. TRACHOMATIS*

Monoclonal Antibody	IgG Isotype	Immunoblot Reaction	Micro-IF Reaction	Neutralization	Dot-Blot Reaction
UM-1	1	L_2,B,E,D	L_2,B,E,D,G,MoPn	$L_2^+D^-G^-$	L_2B,E,D
UM-2	2a	G,F,C	G,F,C,K	$G^+F^-C^-D^-J^-$	F,G
UM-3	1	L_2,E,G,MoPn	L_2,E,G,MoPn	$L_2^-G^-$	-
UM-4	2b	L_2	L_2	$L_2^+D^-J^-A^-G^-$	L_2
UM-5	1	J,H,I,A,C	J,H,I,A,C	$L_2^-D^-J^-$	-
UM-6	2a	A-K,L_2,MoPn,Cp	C,J,I,A	$A^+L_2^-D^-J^-G^-$	J,I,A,C
C11	2b	A-K,L_2	A-K,L_2	$L_2^+A^-G^-B^-F^-$	L_2,B,F,G

* All monoclonal antibodies to MOMP are ELISA positive

"+" Denotes a positive result

"-" Denotes a negative result

TABLE 6

CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO NON-MOMP ANTIGENS OF C. TRACHOMATIS

Monoclonal Antibody	IgG Isotype	Epitope Recognized	Immunoblot	Micro-IF Reaction	ELISA	Dot-Blot Reaction
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	--	-	-

specificity determined by immunoblot, micro-immunofluorescence and ELISA. We were interested in the surface accessibility of these epitopes on native EBs. All Mabs were reacted with native EBs immobilized on nitrocellulose paper in a dot blot apparatus. Figure 15 shows the reactivity of these Mabs against serovars A-K, L_2, and C. psittaci in the dot-blot assay which is a measure of immunoaccessibility as well as epitope density. UM-1 reacted with serovar L_2 and with serovars E, and D to a lesser extent. UM-2 bound strongly to FG and showed some binding with L_2 and D. UM-3 bound weakly while UM-4 reacted strongly to serovar L_2. UM-5 did not bind at all. C11 reacted with serovars L_2, F, G, B, E, and D even though it showed species-specificity by micro-IF and immunoblot. UM-6 bound to epitopes on serovars J, I, A, and C which is similar to its micro-IF reaction. By immunoblot, however, UM-6 has genus specificity. Thus, immunoaccessibility to epitopes on native EBs varies among these antibodies and is substantially more restricted than seen in the micro-IF assay and immunoblot assay.

Table 6 shows the characterization of Mabs directed against non-MOMP antigens. These Mabs were almost all identified by immunoblot since they were ELISA negative. Only the 29 kDa Mab was weakly ELISA positive. They were dot blot negative as well. By immunoblot, the Mabs reactive with 29 kDa, 32 kDa, 57 kDa, and 60 kDa antigens demonstrated species-specificity while the Mabs of 10 kDa and 75 kDa antigens appeared to have genus

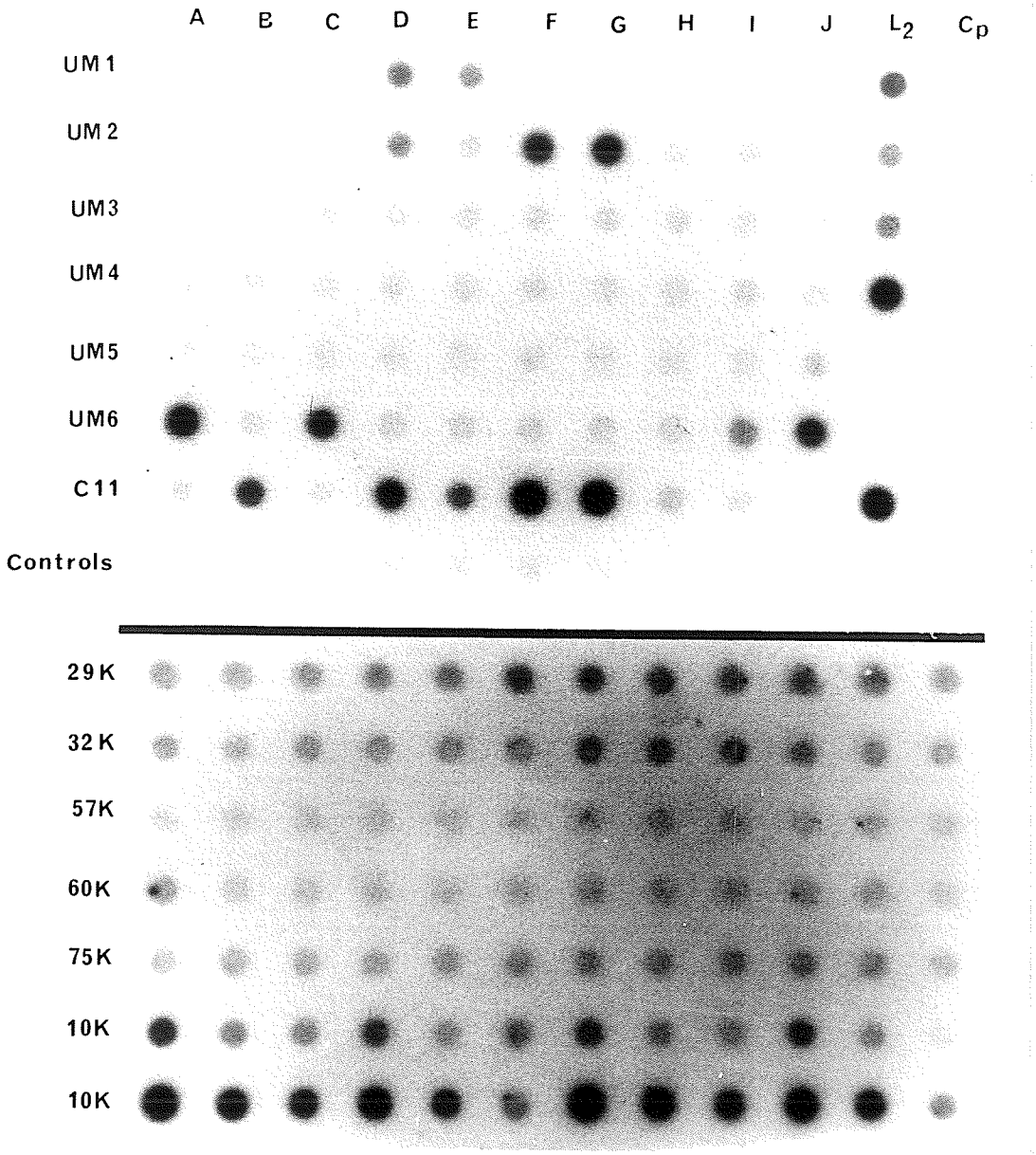


FIGURE 15. Autoradiogram showing the dot-blot reactivities of chlamydial monoclonal antibodies (labelled on the left) against EBs of serovars A-J, L_2 and C. psittaci (shown across the top), detected by ^{125}I-protein A.

specificity. The 70 kDa Mab detected an epitope found on C. trachomatis and C. psittaci but not on the C. trachomatis mouse pneumonitis. The dot-blot reactions were similar to the ELISA reactions for these Mabs. With the exception of UM-7, all Mabs were negative by Micro-IF as well. Mr. Ian Maclean in his Ph.D. dissertation entitled, "Characterization of antigens Chlamydia trachomatis" (thesis in preparation) found that the MOMP and LPS are the major immunoaccessible antigens in the chlamydial outer membrane. A 29 kDa antigen is of limited immunoaccessibility. Antigens of 75kDa, 70 kDa, 60 kDa, 57 kDa and 32 kDa are also found in the outer membrane but are either not immunoaccessible or have very limited surface exposure.

C. Neutralization of Infectivity

i) Screening of hybridoma culture supernatants

For the initial screening of culture supernatants from the 99 hybridomas produced, we used a neutralization assay as previously described (Peeling et al, 1984). Three hybridoma culture supernatants neutralized serovar L_2 infectivity in HeLa 229 cells. These are UM-1, an IgG_1, Mab recognizing a sub-species epitope on MOMP; UM-4, an IgG 2b Mab recognizing a type-specific epitope on MOMP and C11, an IgG 2b Mab recognizing a species-specific on MOMP by immunoblot and micro-IF. These Mabs all bound intensely to L_2 in the dot-blot assay.

As discussed in the literature review, neutralization of C. trachomatis with Mab in previous publications has resulted in considerable variability. Some investigators have been unable to demonstrate neutralization; others have reported that neutralization is strictly complement dependent. We have reported a Mab to MOMP which could neutralize C. trachomatis without the addition of complement. We therefore used these three Mabs to evaluate parameters which could influence the efficiency of neutralization. The results of these experiments are reported in the next section.

ii) Optimizing conditions for neutralization

Our first concern with the neutralization assay was the effect of heat inactivation on chlamydial viability since these organisms have remarkable thermal instability. The thermal inactivation in PBS at 37°C of C. trachomatis is shown in Figure 16. Approximately half the inoculum was inactivated after 60 minutes at 37°C. The presence of 10 ug/ml BSA in the inoculum was slightly protective but normal mouse IgG at the same concentration essentially maintained the viability of inoculum for 60 minutes.

The optimal amount of normal mouse IgG required to maintain EB viability was next determined (Figure 17). An optimal concentration of 10 ug/ml of IgG was "protective" against thermal inactivation at 37°C for 30 minutes. Thus all subsequent neutralization assays were

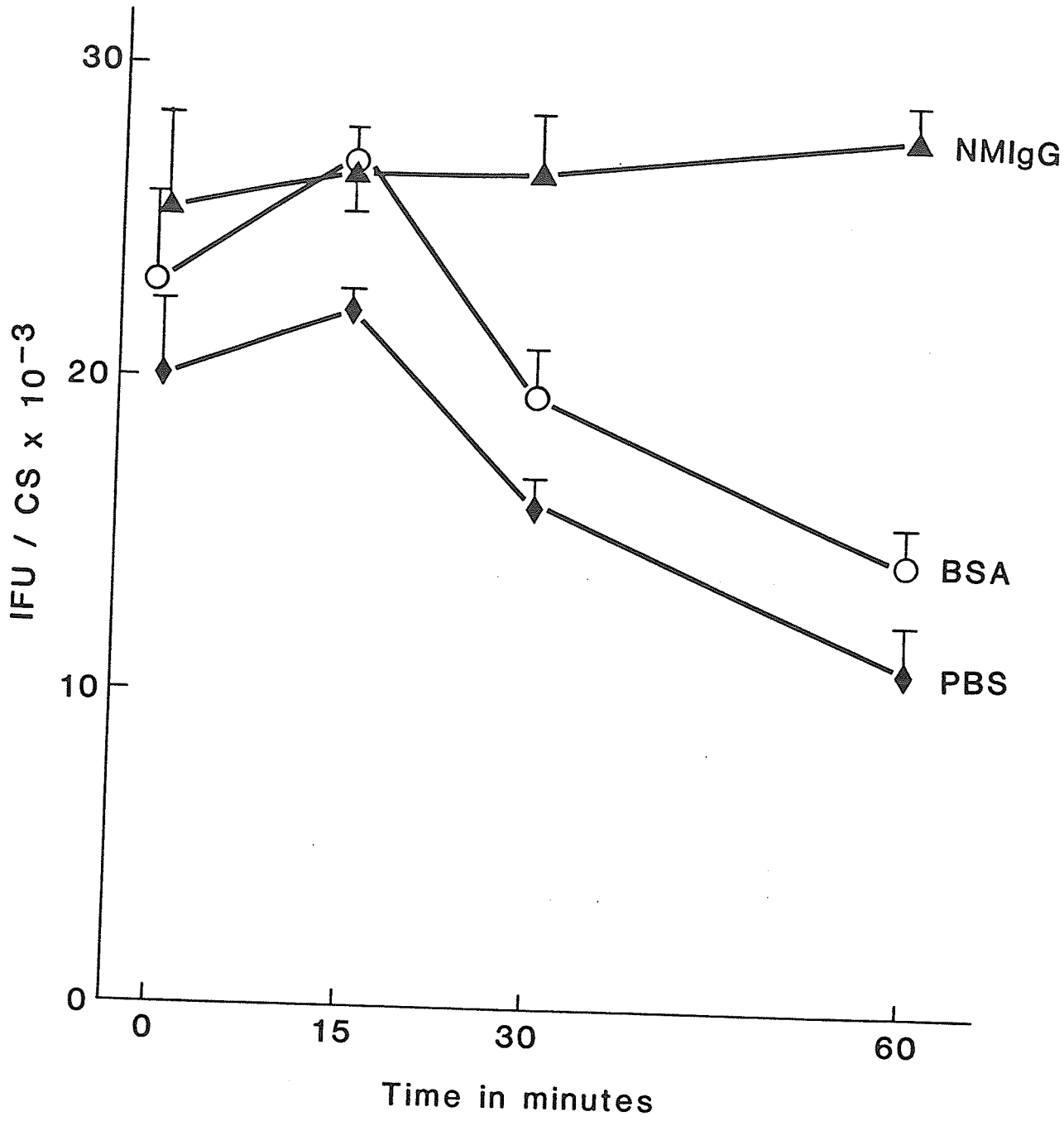


FIGURE 16. Thermal inactivation of C. trachomatis serovar L_2 EBs. Purified EBs were incubated at 37°C with an equal volume of 0.01 M PBS, 1% BSA in PBS and 10 ug/ml of NMIgG in PBS. Aliquots were taken at 0', 15', 30' and 60' and their infectivity determined by counting inclusion-forming units on HeLa 229 coverslip (IFU/cs) cultures.

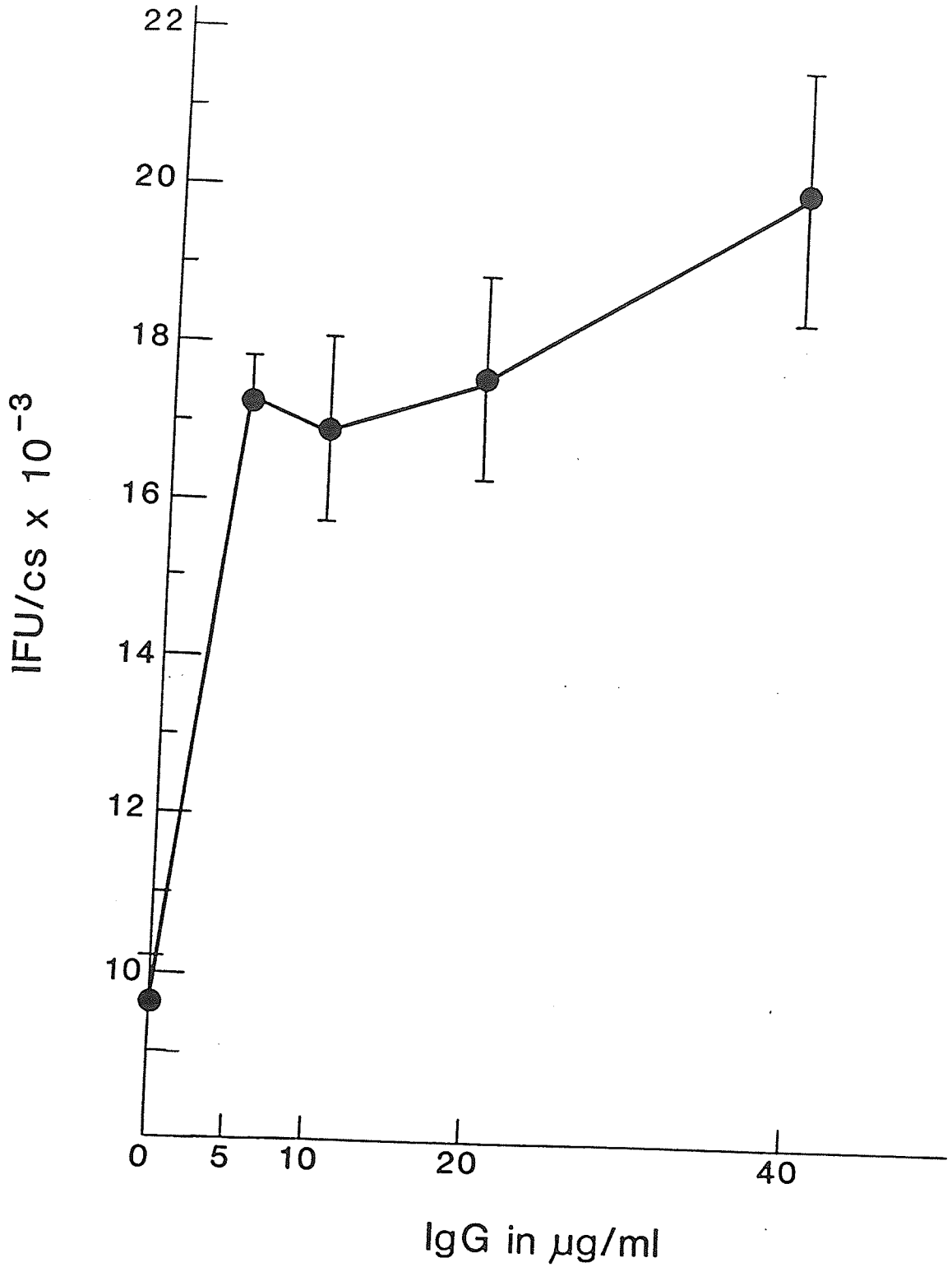


FIGURE 17. Protective effect of varying concentrations of normal mouse IgG on the infectivity of C. trachomatis serovar L_2 EBs. EBs were incubated at 37°C for 30' with an equal volume of 5, 10, 20, 40 ug/ml of NMIgG of C. trachomatis serovar L_2 EBs. Infectivity was expressed as IFU per coverslip culture

performed with 10 ug/ml of normal mouse IgG in PBS as antibody diluent.

The traditional medium for suspension of EBs is SPG. Thus, SPG was compared with PBS as antigen diluent in a neutralization assay of L_2 with antibody C11. Neutralization was better detected when SPG rather than PBS was used as diluent (Fig. 18). The C11 antibody was diluted in 10 ug/ml of normal mouse IgG, so heat inactivation was not a factor here.

Having established suitable diluents for both antigen and antibody, we next determined the optimal concentration of antigen and antibody to use in the assay. For a 12 mm coverslip culture, 5×10^2 - 10^5 IFU per coverslip are just within counting limits. Figure 19 shows the effect of using excessive antigen concentrations against 4-fold dilutions of IgG from Mab UM-4. At high antigen concentration (9×10^4 IFU/CS), not only were the coverslips difficult to count because the inclusions were so close together, but the extent of neutralization was less than at lower antigen concentration (6×10^4 IFU/CS). At high concentrations of antibody, neutralization was also less effective, consistent with a prozone effect. For an inoculum of 60,000 IFU per coverslip, the most effective neutralization of infectivity for Hela 229 cells was between 0.63-2.5 ug/ml of UM-4 IgG, whereas 10 ug/ml UM-4 IgG was less effective. Thus we used a range of IgG concentrations from 0.04 to 40 ug/ml and a

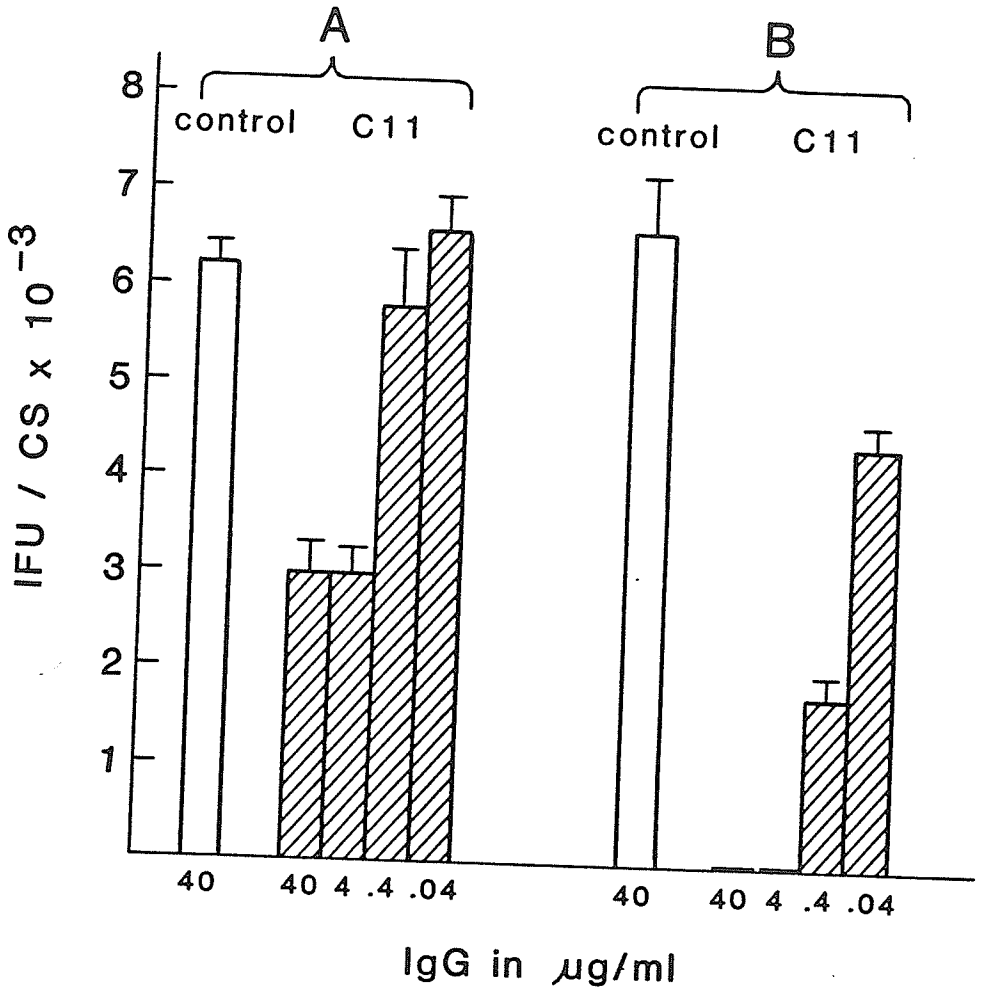


FIGURE 18. Effect of antigen diluent on neutralization of C. trachomatis infectivity. Purified serovar L_2 EBs were suspended in PBS (A) or SPG (B) and incubated with 10-fold dilutions of Mab C11 IgG in PBS (containing 10 ug/ml of NMIgG) at 37°C for 30 min. NMIgG was used as control. Residual infectivity was determined by counting inclusion forming units per coverslip (IFU/cs) culture of HeLa 229 cells.

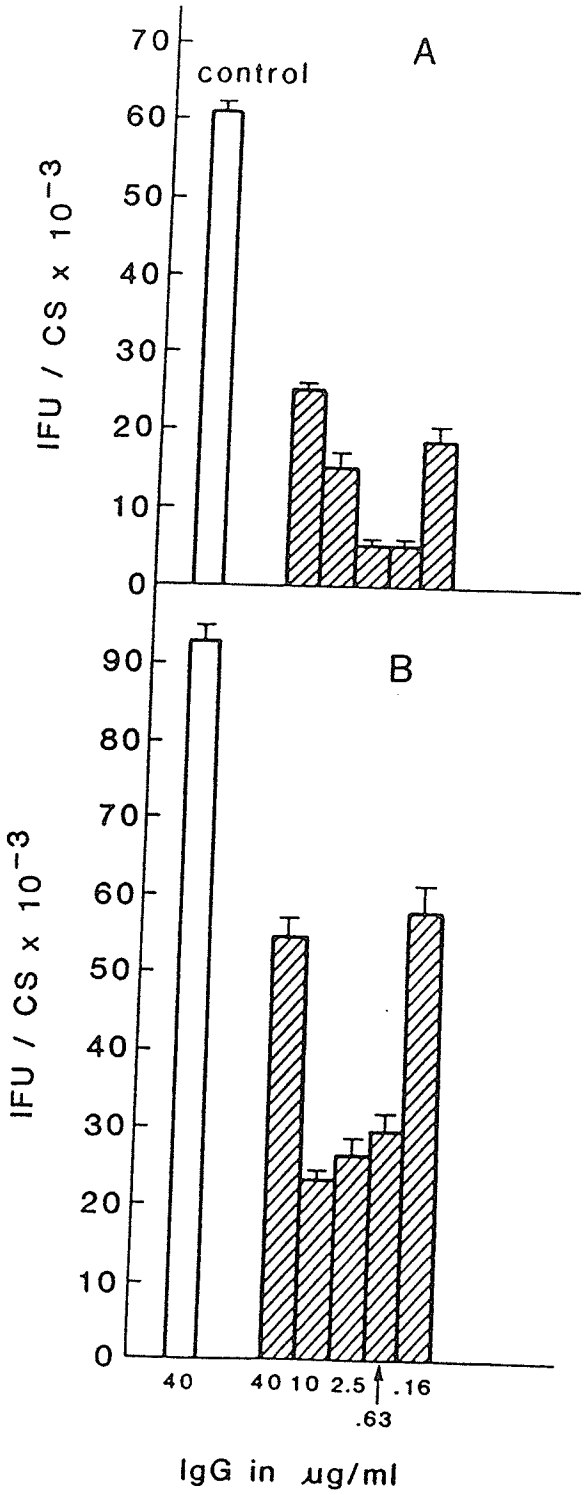


FIGURE 19. Effect of antigen and antibody concentration on neutralization of C. trachomatis infectivity. Two concentrations of serovar L_2 EBs (A and B) were incubated with four concentrations of Mab IgG at 37°C for 30 min. Residual infectivity in HeLa cells was expressed as IFUs per coverslip

concentration of antigen was between 10^3-10^4 IFU per coverslip to achieve optimal neutralization (refer to Figure 23).

We next evaluated the effect of temperature, duration of incubation and volume of reactants as variables affecting the neutralization reaction. Table 7 shows that neutralization was not as effective when incubated at 4°C for 60' as compared to 37°C for 30 minutes. The smaller reaction volume of 360 ul was better than 1 ml in the neutralization assay (Table 8).

To assess the possibility that the HeLa cell cycle may affect neutralization, the growth cycle of HeLa 229 cells was synchronized using a double thymidine block. The % of neutralization in duplicate assays was unchanged whether the cells were synchronized or not (Table 9).

In summary, the factors which affect the neutralization assay are:

1. Thermal inactivation of EBs
2. Volume reaction
3. Temperature of incubation
4. Antigen and antibody concentrations

iii) Screening of Mabs using purified IgG

Having established the optimal conditions for the in vitro neutralization assay, we evaluated the 14 Mabs listed in Table 5 and 6 for neutralizing activity. Based on their surface exposure as determined by

TABLE 7

EFFECT OF INCUBATION TEMPERATURE ON NEUTRALIZATION

Mab	in ug/ml	% Neutralization	
		37°C/30'	4°C/60'
UM-4	40.0	70	40
	4.0	58	21
	.4	52	0
	.04	51	0

Purified serovar L_2 EBs were incubated with 10-fold dilutions of Mab UM-4 IgG at 37°C for 30' or 4°C for 60'. Residual infectivity was determined by counting inclusion forming units (IFUs) on coverslip cultures of HeLa 229 cells. Percentage neutralization was calculated as % reduction in IFUs compared to NMIgG controls under similar incubation conditions.

TABLE 8

EFFECT OF VOLUME OF REACTION ON NEUTRALIZATION

Mab Designation	IgG in ug/ml	% Neutralization Volume of Reaction 360 ul	1 ml
UM-4	40.0	70	56
	4.0	58	38
	0.4	52	28
	0.04	51	16

180 ul of purified serovar L_2 EBs were combined with an equal volume of Mab UM-4 IgG in concentration as shown. A duplicated set of reaction mixes were prepared as described above except that the total final volume was diluted to 1 ml with a 50/50 mixture of SPG + PBS. These reaction mixes were all incubated at 37°C for 30 min. Residual infectivity was determined by counting inclusion forming units per coverslip (IFU/cs) cultures of HeLa 229 cells. Percentage of neutralization was expressed as reduction in IFU/cs in Mab treated EBs as compared to NMIgG treated EBs.

TABLE 9

EFFECT OF CELL CYCLE STATUS ON NEUTRALIZATION

Mab IgG in ug/ml	% Neutralization	
	Synchronous Culture	Asynchronous Culture
4.0	55	58
0.4	36	44
0.04	40	45

Purified serovar L_2 EBs were incubated with UM-1 IgG at 37°C for 30 min. Residual infectivity for HeLa 229 cells which were synchronized by double thymidine block were compared with that for asynchronous HeLa cell cultures.

dot-blot and micro-IF, the Mabs were reacted against different serovars in the neutralization assay.

UM-1, which recognizes serovars L_2, B, E and D, was tested against serovars L_2, D and G. It only neutralized L_2 to which it bound most intensely in the dot-blot assay. UM-2 bound serovars F and G strongly in the dot-blot but also reacted with serovars C and K in micro-IF. It reduced infectivity of serovar G by only about 50% and was not neutralizing for serovars F, C, D and J. UM-3, did not bind any epitopes on native EBs and did not neutralize serovars L_2 and G. UM-4, a Mab recognizing a type-specific epitope on serovar L_2 by all the assays neutralized serovar L_2 strongly but not serovars D, J, A, and G. Similar to UM-3, UM-5 did not recognize any epitopes on any EBs in the dot-blot assay and also did not neutralize the infectivity of any of the serovars tested (L_2, D, and J). UM-6, the genus-specific Mab which only recognized serovar J, I, A, and C in dot-blot, neutralized the infectivity of servar A and not that of L_2, D, J and G. C11, bound serovar L_2 strongly in the dot-blot assay and neutralized serovar L2 and not A or G even though it showed some binding to G in the native EBs Fig. 20, Tables 10 and 11).

None of the Mabs specifying non-MOMP epitopes were positive in the dot-blot assay except the Mabs to the 10 kDa and 29 kDa antigens. All of those Mabs gave

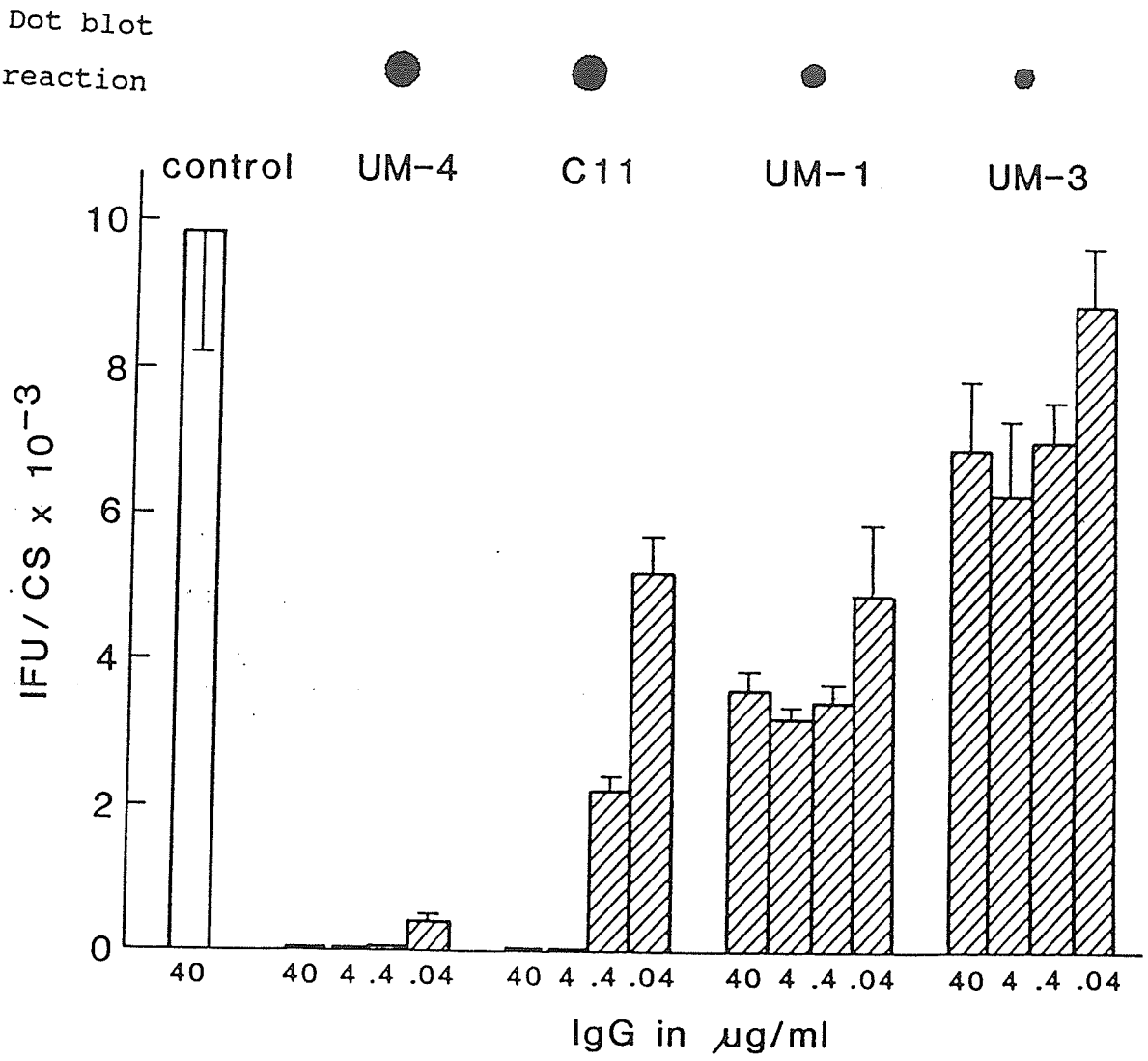


FIGURE 20. Neutralization of infectivity of C. trachomatis of serovar L_2 EBs. Purified serovar L_2 EBs were incubated with IgG from normal mouse serum, Mab UM-1, UM-3, UM-4 and C11 at 37°C for 30 min. Residual infectivity for HeLa 229 cells was determined by counting inclusion forming units per coverslip culture. Neutralization of infectivity was compared with dot-blot reactivities of these Mabs to native EBs.

TABLE 10

NEUTRALIZATION OF INFECTIVITY OF SEROVAR A

IgG (ug/ml) \ Mab	% Neutralization		
	UM-6	C11	UM-4
40.0	63	0	18
4.0	28	0	0
.4	33	8	18
.04	34	13	0

Purified serovar A EBs were incubated with IgG from normal mouse serum, Mabs UM-2, C11 and UM-4 at 37°C for 30 min. Residual infectivity for HeLa 229 cells was determined by counting inclusion forming units per coverslip. Neutralization expressed as % reduction in inclusion forming units by Mabs compared to normal mouse controls.

TABLE 11

NEUTRALIZATION OF INFECTIVITY OF SEROVAR G

IgG (ug/ml) \ Mab	% Neutralization		
	UM-2	C11	UM-4
40.0	55	46	30
4.0	54	36	0
.4	51	24	0
.04	30	34	0

Purified serovar G EBs were incubated with IgG from normal mouse serum, Mabs UM-2, C11 and UM-4 at 37°C for 30 min. Residual infectivity for HeLa 229 cells was determined by counting inclusion forming units per coverslip culture. Neutralization expressed as % reduction in inclusion forming units by Mabs compared to normal mouse controls.

negative reactions in the neutralization assay for serovar L_2.

In conclusion, only Mabs specifying epitopes on MOMP neutralized infectivity in vitro. The strongest neutralization reactions were type-specific. Subspecies-specific Mabs appear to neutralize with less intensity. The capacity for neutralization correlated best with the intensity of binding in the dot-blot reaction.

2. In vivo assay: Mouse toxicity prevention test

Zhang et al (1986) reported that Mabs to MOMP which were protective in the monkey eye challenge model were also protective in the mouse toxicity prevention test. We therefore evaluated Mab, UM-1, which neutralized serovar L_2 but not D in vitro Fig. 21, for mice protection in the MTPT. Table 12 shows the results in mice given lethal doses of EBs from serovar L_2 and D which had been reacted with saline or Mab, UM-1. UM-1 was protective for 4 of 6 mice injected with 10^7 antibody treated EBs. All the mice injected with equal amounts of EBs treated with saline died in 24 hours. UM-1 did not protect mice from toxic death with serovar D EBs. Thus, in vitro antibody-mediated neutralization correlates with toxin neutralization.

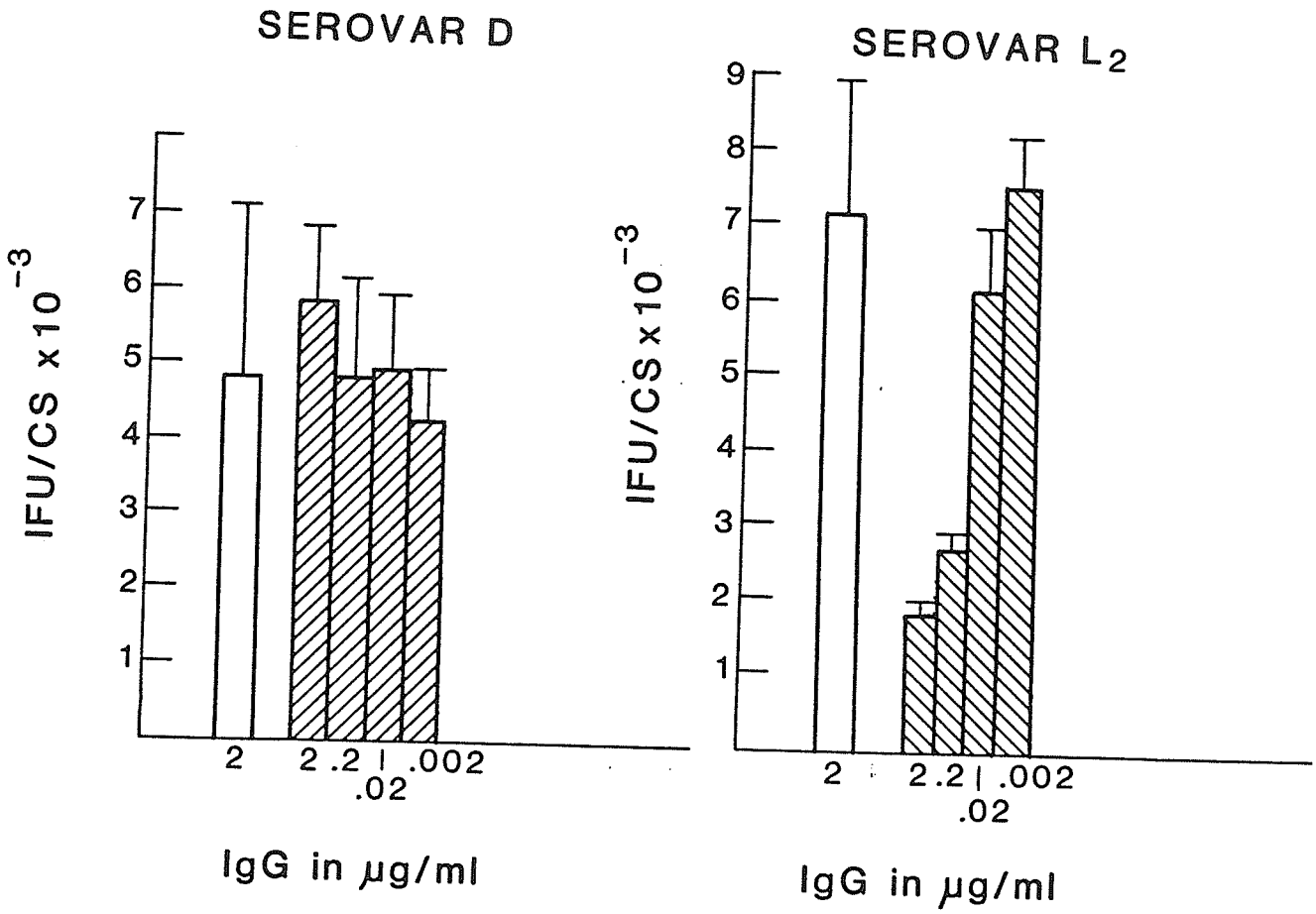


FIGURE 21. Neutralization of C. trachomatis serovars L_2 and D infectivity by Mab, UM-1. Purified serovar L_2 or serovar D EBs were incubated with NMIgG (open bars) or UM-1 IgG (hatched bars) at 37°C for 30 min. Residual infectivity for HeLa 229 cells was determined by counting inclusion forming units per coverslip (IFU/cs) culture.

TABLE 12.

MOUSE TOXICITY PREVENTION TEST

Dose (IFU)	Mouse Toxicity (No. Dead/ No. Injected)			
	Serovar D		Serovar L_2	
	+ Saline	+ UM-1	+ Saline	$+^{2}$UM-1
5×10^6	1/6	0/6	0/6	0/6
1×10^7	3/6	4/6	4/6	0/6
5×10^7	4/6	6/6	N.D.	N.D.
1×10^8	6/6	4/6	N.D.	N.D.

4 week old Balb/c mice were given intravenous injections of C. trachomatis serovar L_2 or D EBs pre-treated with saline or Mab UM-1 IgG for 60 min at room temperature. Death in 24 hours associated with each treatment was recorded.

3. Mechanism of Neutralization

We next evaluated the stage of infection at which antibody-mediated neutralization occurred Serovar L_2 was used throughout these experiments. All three neutralizing Mabs, UM-1, UM-4, and C11 were used in the attachment and endocytosis assays. Mab UM-4 was used in the immunofluorescence assay when it became necessary to follow the intracellular fate of antibody-coated EBs.

i) Inhibition of Attachment

^{14}C-labelled EBs were incubated with purified IgG of Mabs UM-1, UM-4, C11 and of normal mouse IgG at 37°C for 30 minutes. The reaction mixes were layered onto HeLa cell monolayers and allowed to adsorb for 2 hours at room temperature. After 30, 60 and 120 minutes, monolayers were washed and lysed in 2% SDS. Total cell-asociated ^{14}C cpms from each lysate represented the number of EBs from each treatment that had attached and/or been endocytosed. Fig. 22 shows the kinetics of attachment to HeLa cell monolayers of neutralized EBs compared to control EBs. The mean of each treatment at each time point was compared to the control preparation and analyzed by the analyses of variance (ANOVA). There were no statistically significant differences among the treatments. The slope of the graphs were also analyzed by an analysis of co-variance and again no statistically significant differences were observed in the rate of attachment of these antibody-coated EBs compared to the

control. Thus Mabs which neutralized L_2 did not interfere with cell association.

ii) Inhibition of Endocytosis

To assay the rate of endocytosis of ^{14}C-labelled EBs into HeLa cells, a modified procedure of the attachment assay was used. Heparin, at a concentration of 100 units/ml is known to release at least 80% of serovar L_2 EBs attached to HeLa cell monolayers. Thus, after EB adsorption, monolayers were treated with heparin for 30 minutes at room temperature to release attached but unendocytosed EBs. Fig. 23 shows the kinetics of endocytosis of antibody-coated EBs into HeLa cells over two hours. Again, the mean of radioactive counts for each treatment at each time point was compared to the control in an analysis of variance. There was a difference between the uptake of EBs treated with Mab, C11 at 60 minutes compared to that of the control. However, as there were no statistical differences between their uptake at 30 minutes and 120 minutes, it appears that this could be just a spurious result. In the analyses of co-variance, the slopes of the uptake of these Mab-coated EBs were not different from that of the control. Thus Mabs which neutralized serovar L_2 infectivity did not impede endocytosis.

FIGURE 22. Attachment of antibody-treated C. trachomatis serovar L_2 EBs to HeLa cells. ^{14}C-labelled serovar L_2 EBs were incubated with IgG of Mabs C11 (1), UM-1 (2), UM-4 (3) or normal mouse (4) at 37°C for 30 minutes. The reaction mixes were allowed to adsorb to HeLa 229 cells in coverslip cultures for 120 minutes at 4°C. The coverslip cultures were washed and lysed with 2% SDS. Radioactivity of the cell lysates were determined in a scintillation counter.

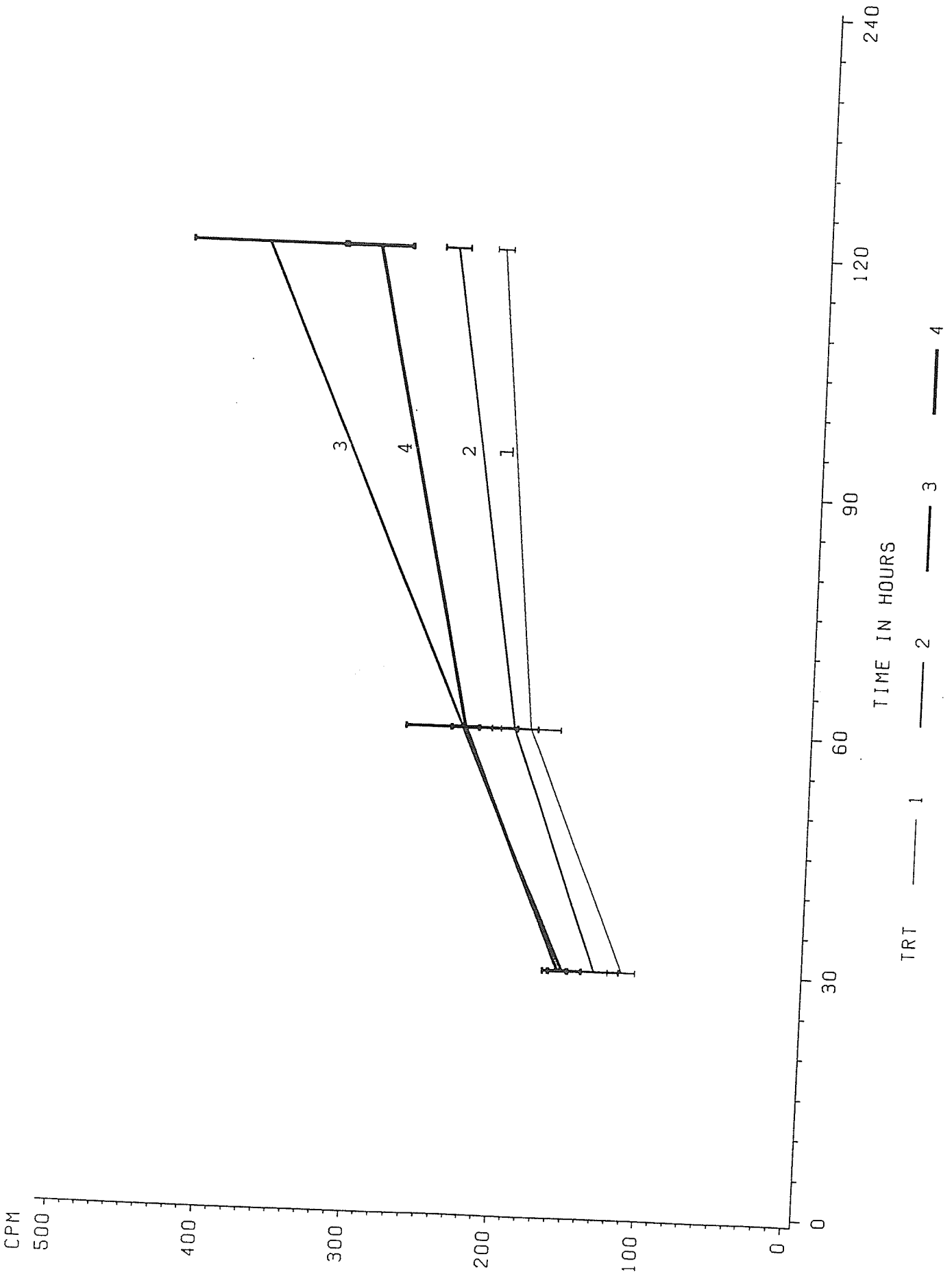
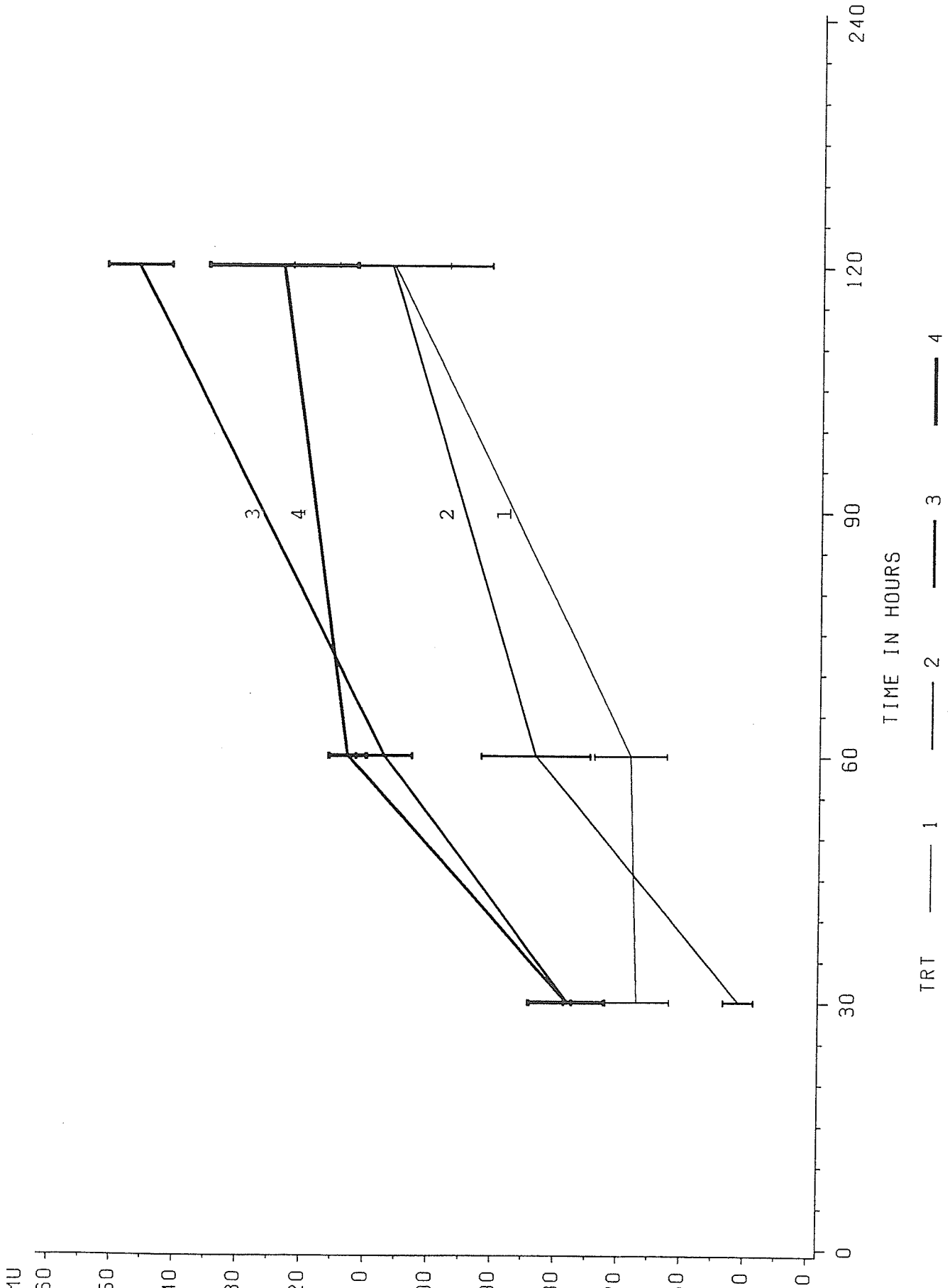

CPM
500
400
300
200
100
0
0
30
60
90
120
240
TIME IN HOURS
TRT 1 2 3 4
3
4
2
1

FIGURE 23. Endocytosis of antibody-treated C. trachomatis serovar L_2 EBs into HeLa cells. ^{14}C-labelled L_2 EBs were incubated with IgG of Mabs C11 (1), UM-1 (2), UM-4 (3) or normal mouse (4) at 37°C for 30 min. The reaction mixes were allowed to adsorb to HeLa 229 cells in coverslip cultures for 120 min at room temperature. Unendocytosed but attached EBs were eluted by treatment wth 100 units/ml of heparin. Coverslips were washed and radioactivity in cell lysates were determined in a scintillation counter

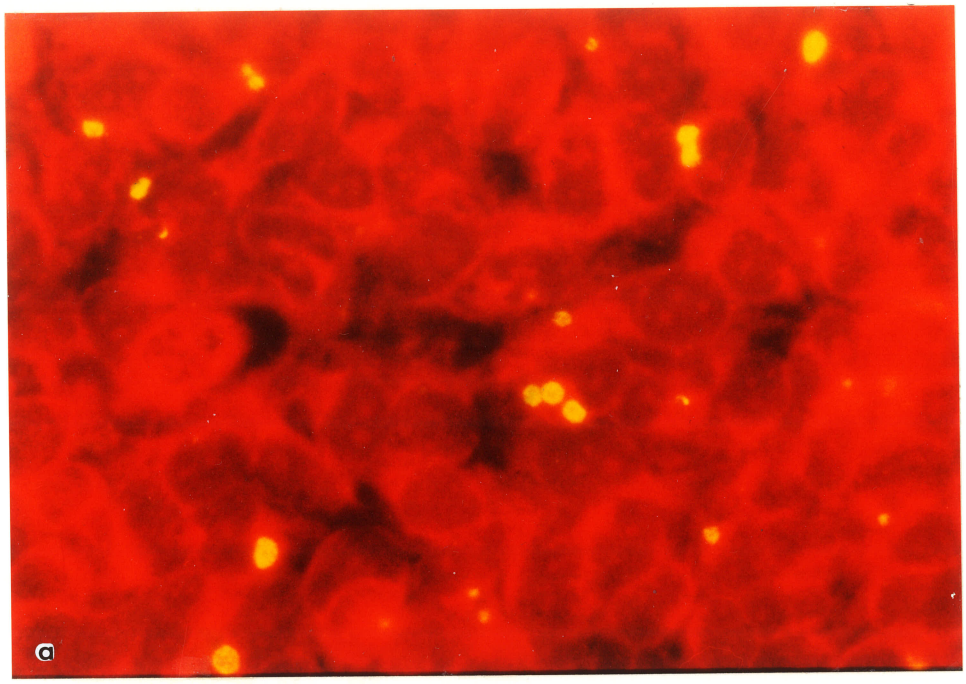

CPMU
160
150
140
130
120
110
100
90
80
70
60
50
40
0
30
60
90
120
240
TIME IN HOURS
TRT 1 2 3 4
1
2
3
4

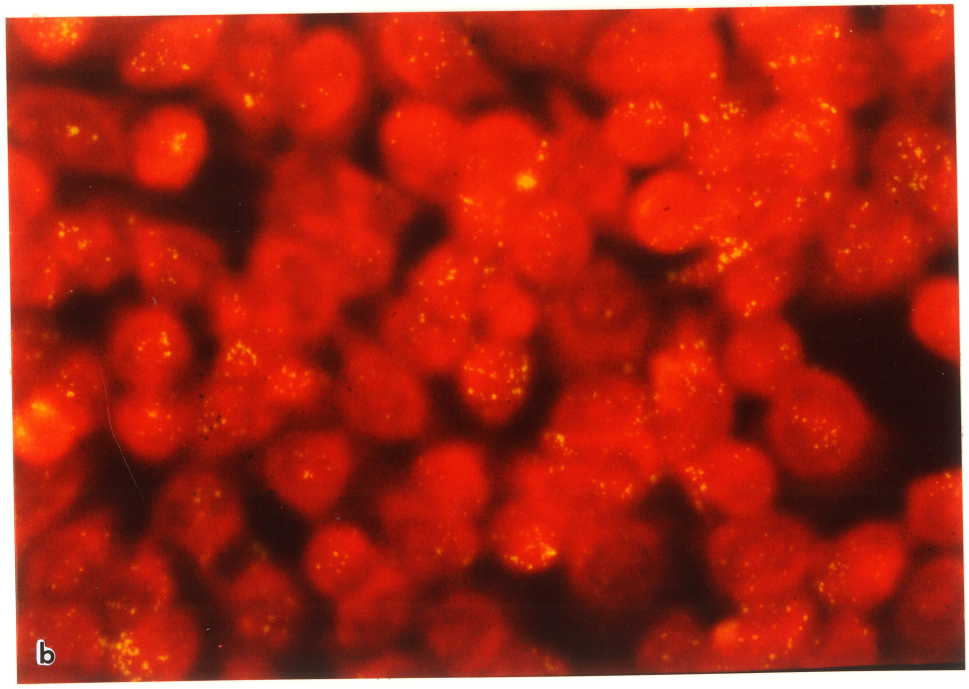
iii) Immunofluorescent staining of antibody-coated EBs

Since neutralizing Mabs did not appear to affect the attachment and entry phases of serovar L_2 EBs into HeLa cells, we used fluorescein-conjugated anti-mouse IgG to follow the intracellular fate of these antibody-coated EBs at 2, 4 and 20 hours post-infection. This method is not quantitative, thus we cannot discount the possibility that some antibody-coated EBs had been destroyed by phagolysosomal fusion. Fig. 24 shows the presence of antibody-coated EBs, stained with fluorescein-conjugated anti-mouse IgG, in infected HeLa cell monolayers up to 20 hours post-infection. At this time, the EBs in the control preparation had differentiated into RBs and small chlamydial inclusions were evident. These control monolayers were stained with fluorescein-conjugated anti-chlamydial antibody. Thus these experiments show that in HeLa cell monolayers infected with EBs coated with Mab IgG which neutralized infectivity, the organism persisted intracellularly morphologically intact for at least 20 hours. At this stage, we were unable to define the exact mechanism for intracellular neutralization produced by anti-MOMP antibody. Later on we developed biochemical methods to measure EB to RB differentiation and thus we were able to further characterize the mechanism of antibody neutralization.

Prior to developing these techniques, we took a different approach to characterize the role of outer membrane proteins in the pathogenesis of eucaryotic cell

FIGURE 24. Immunofluorescence staining of antibody-coated EBs in HeLa cells. Serovar L_2 EBs were incubated with normal mouse IgG or Mab UM-4 IgG at 37°C for 30 min. Residual infectivity in these mixtures were monitored in HeLa cells at 20 hours post-infection. (a) Control EBs were stained with fluorescein conjugated anti-chlamydial IgG. Antibody-coated EBs were stained with fluorescein conjugated anti-mouse IgG (b)


a


b

infection. The results of these experiments will be described before returning to studies of antibody mediated neutralization since they provided insight into the potential mechanism of neutralization.

D. Trypsin Studies

To explore further the structure-function relationships of outer membrane proteins in the pathogenesis of C. trachomatis eucaryotic cell infection, the organism was treated with trypsin to digest outer membrane proteins. Structural and biologic effects of proteolysis of EB outer membrane proteins were then analyzed.

1. Enhancement of infectivity

Purified C. trachomatis EBs of serovar L_2 and D were treated with trypsin or buffer alone. Samples for infectivity assays were collected after 15 minutes, 30 minutes, 1 hour, 2 hours, and 4 hours. Fig. 25 shows the effect of trypsin on infectivity of serovars L_2 and D in HeLa cells. The time related decrease in inclusions in the control preparations was due to the thermal inactivation of EBs on prolonged heat exposure. Serovar L_2 EBs appeared less heat labile than serovar D EBs. Proteolysis resulted in more than 200% increase in infectivity of EBs of both serovar L_2 and D when exposure to trypsin lasted 15 to 60 minutes. For serovar D, enhancement was evident even after the control EBs were completely heat inactivated.

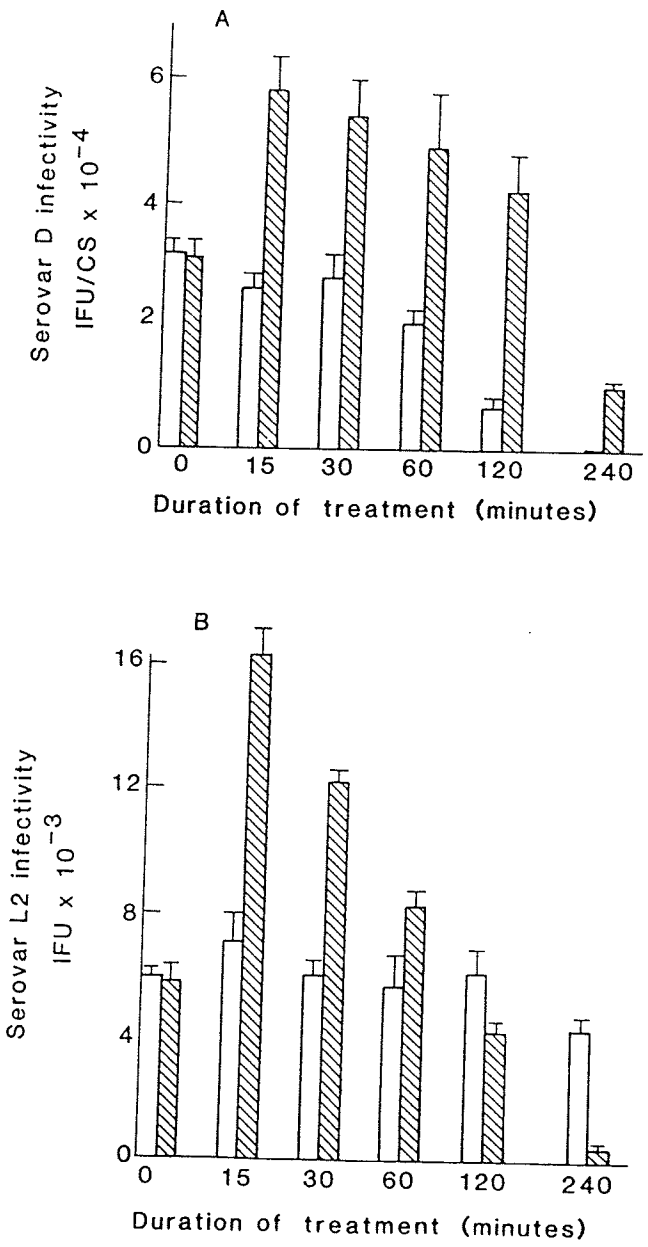


FIGURE 25. Effect of trypsinization on the infectivity of C. trachomatis EBs. (A) serovar L_2, (B) serovar D. EBs were treated with 1 mg/ml of trypsin (shaded bars) or Tris buffer (open bars) at 37°C for the times indicated. Infectivity was determined in HeLa 229 coverslip cultures and expressed as IFUs per coverslip

Because we had more immunological reagents for L_2 proteins, and because L_2 EBs exhibited greater resistance to heat inactivation, we used serovar L_2 EBs for subsequent experiments.

We used three concentrations of trypsin to determine the optimal concentrations of trypsin enhancement of L_2 infectivity (Fig. 26). Infectivity of trypsinized EBs was expressed as a percent of the controls. Less enhancement of infectivity was seen with lower concentrations of trypsin (232% versus 163% and 151% at 1 mg/ml, 100 ug/ml and 10 ug/ml of trypsin respectively). Infectivity decreased with prolonged trypsinization. Trypsin treatment of EBs at 1 mg/ml for four hours resulted in a > 90% reduction of infectivity. After 8 hours of trypsinization, EBs were completely inactivated. Maximal enhancement and reduction of infectivity were evident at the highest concentration of trypsin tested. We were intrigued by the infectivity enhancing effect of trypsin and pursued experiments to define its mechanism of action.

2. Structural Effects of trypsinization on outer membrane proteins

C. trachomatis serovar L_2 EBs were trypsinized for 15 minutes at 37°C for 30 minutes. Trypsinized EBs were washed with PBS and surface labelled by the ^{125}I-lactoperoxidase method. Control EBs were radio-labelled without trypsinization. These EBs were then resolved in SDS-PAGE.

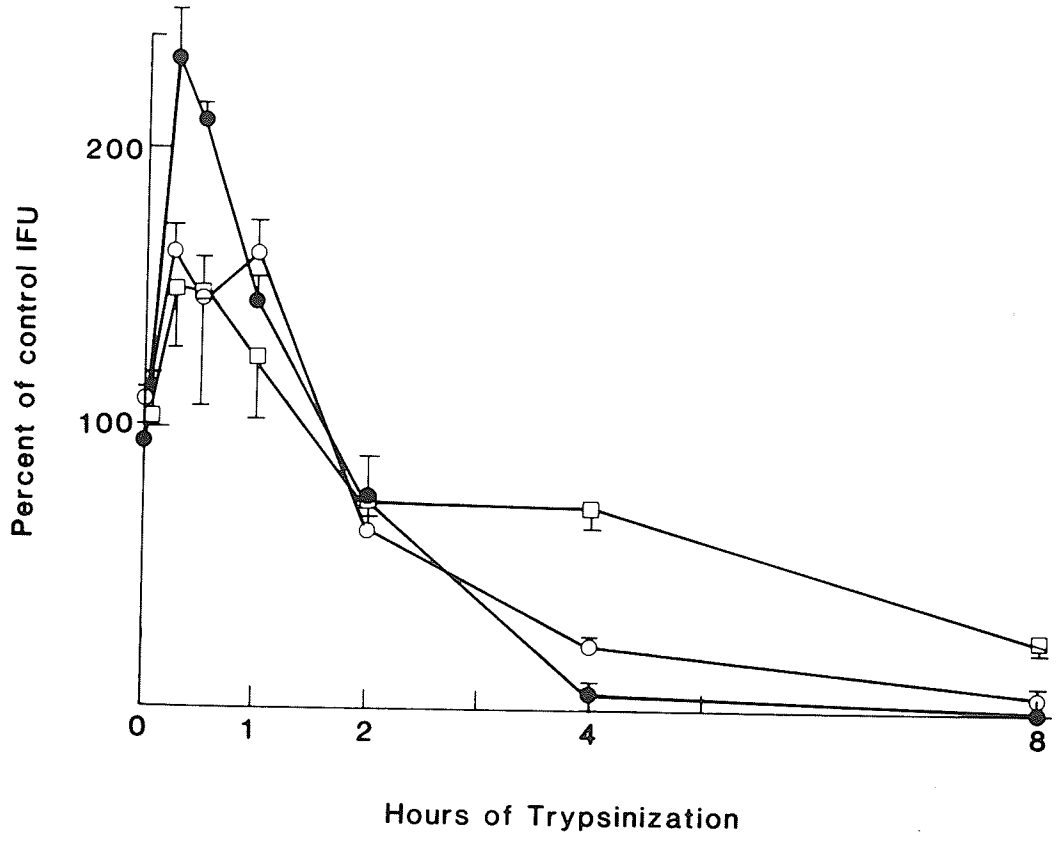


FIGURE 26. Effect of trypsin concentration on infectivity of C. trachomatis serovar L_2 EBs. EBs were treated with 1 mg/ml (●), 100 ug/ml (O), 10 ug/ml (□) of trypsin or Tris buffer control at 37°C for the times indicated. Infectivity was determined in HeLa 229 coverslip cultures and expressed as a percentage of inclusion forming units (IFU) in control cultures

Fig. 27 shows the effect of trypsin digestion on outer membrane proteins of serovar L_2 EBs. The most prominent surface-iodinated molecules on native EBs are MOMP and the 75 kDa proteins (lane 1). Both these antigens were cleaved from the EB surface following trypsinization (lane 2). Three major breakdown products of molecular mass 35 kDa, 25 kDa, and 10 kDa were seen after 15 minutes of proteolysis.

3. Mechanism of enhancement

We considered five potential explanations for trypsin enhancement of C. trachomatis infectivity: (1) dispersion of aggregated EBs in the inoculum, (2) enhancement of attachment, (3) enhancement of endocytosis, (4) enhancement of inhibition of phagolysosomal fusion and (5) enhancement of intracellular differentiation of EBs to reticulate bodies (RBs).

Aggregation of EBs

Electron microscopic examination of EBs treated with 1 mg/ml trypsin overnight showed no apparent ultrastructural alteration as determined by negative staining. Examination of both buffer treated and trypsinized EBs showed that particles from both preparations were highly dispersed without apparent clumping.

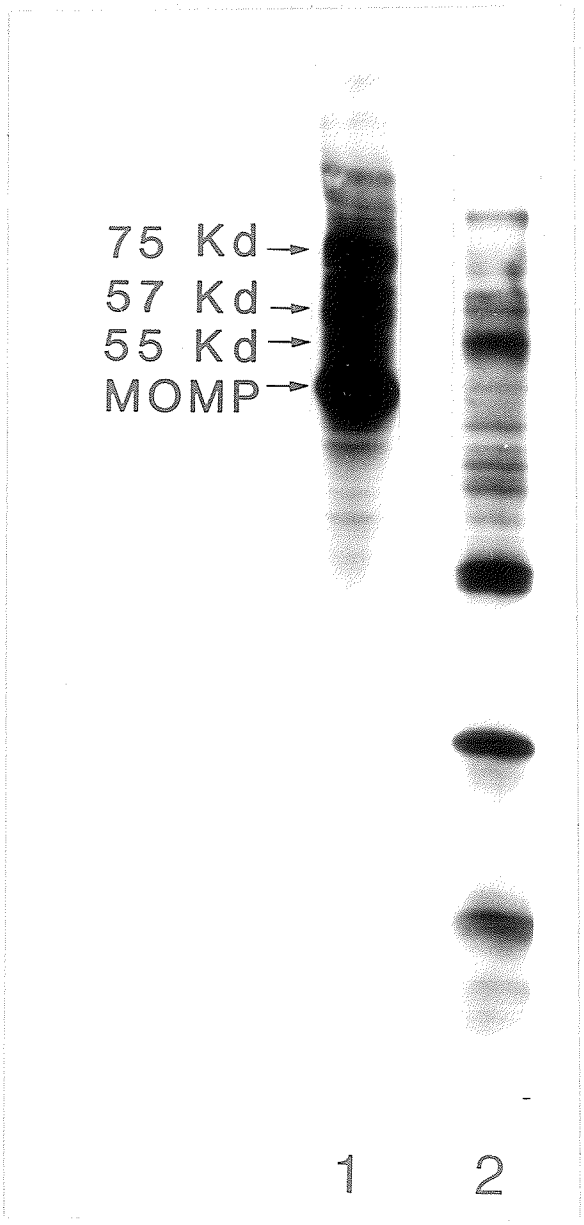


FIGURE 27. Autoradiogram of C. trachomatis of serovar L_2 EBs proteins surface-iodinated by the ^{125}I lactoperoxidase method and resolved on SDS-PAGE Lane 1 shows control untreated EBs. Lanes 2 shows EBs treated with 1 mg/ml of trypsin for 15 min

HeLa Cell Binding and Endocytosis

^{14}C labelled L_2 EBs (50 ug protein) were treated with buffer or with trypsin (1 mg/ml) for 15 minutes and binding determined after interaction with HeLa cells for 30 minutes, 60 minutes, and 2 hours. The kinetics of attachment are shown in Figure 28. The extent and rate of association with HeLa cells for trypsinized EBs was not significantly different than that of untrypsinized EBs ($p > 0.05$).

Purified EBs treated with Tris-saline buffer or with trypsin for 30 minutes were allowed to adsorb to HeLa 220 monolayers at room temperature for 2 hours. Attached but non-endocytosed EBs were released with heparin from one-half the coverslip cultures. The percent of EBs endocytosed into HeLa cells with and without heparin treatment is shown in Table 15. Approximately 40% of attached CPMs were resistant to heparin dissociation and were assumed to represent endocytosed EBs. The proportion of organisms of EBs endocytosed were not affected by treatment of serovar L_2 EBs with trypsin compared to control EBs treated with buffer.

In summary, the following observations were made from trypsin treatment of servoar L_2 EBs. Trypsin has a biphasic effect on chlamydial infectivity. Limited proteolysis enhanced infectivity. MOMP was cleaved into three major fragments within 15 minutes of proteolysis. Trypsinization did not appear to alter attachment and

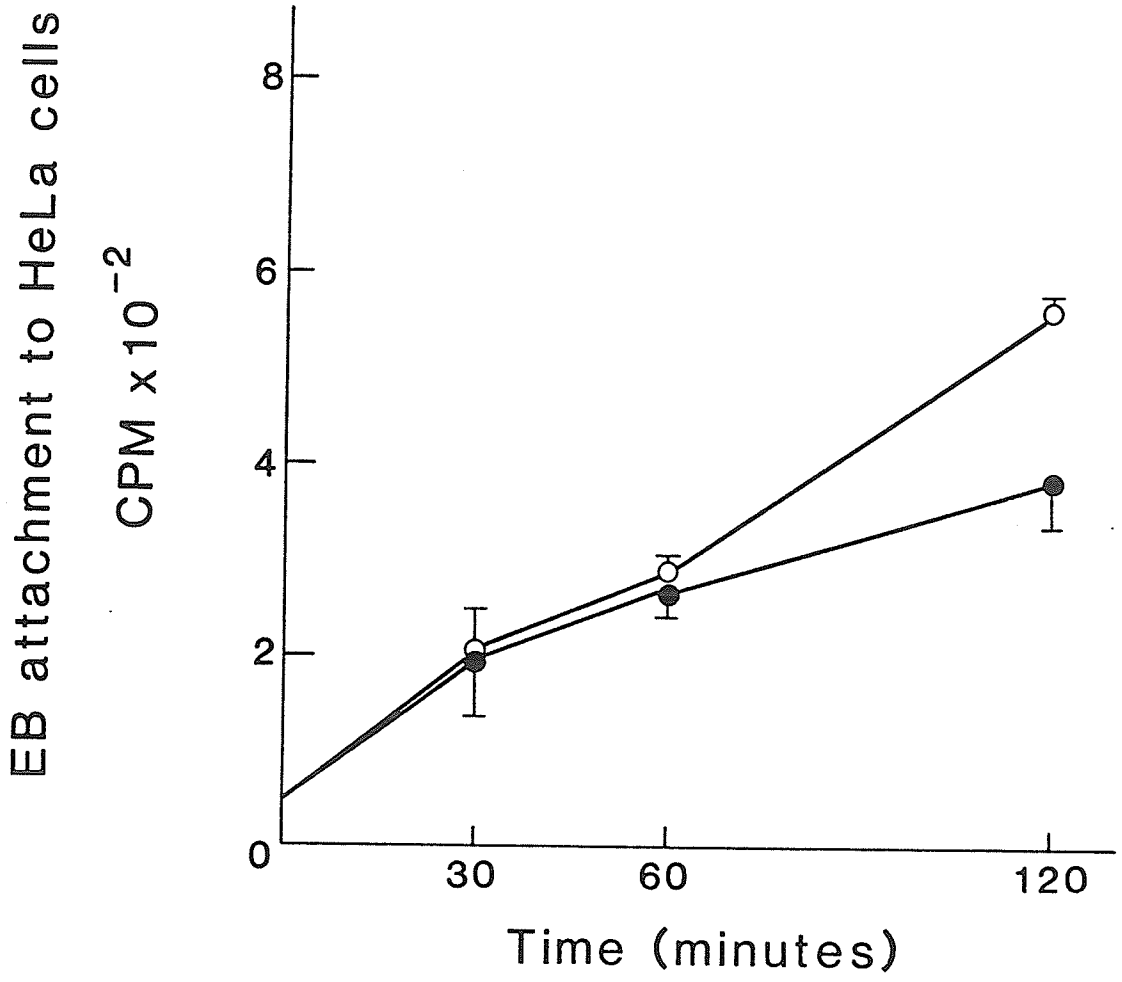


FIGURE 28. Effect of trypsinization on attachment of serovar L_2 EBs. ^{14}C-labelled EBs treated with 1 mg/ml of trypsin (●) or Tris buffer (○) were allowed to bind HeLa cells on coverslips at 4°C. At the times indicated, the coverslip cultures were washed and lysed with 2% SDS. Radioactivity of the lysates was determined in a scintillation counter

TABLE 13

Effect of trypsinization on endocytosis

Trypsin (1 mg/ml)	Total Cell Associated CPMs	Heparin Resistant Cell Associated CPMs	% Endocytosed
-	1931 ± 267	744 ± 85	39%
+	1538 ± 172	591 ± 68	38%

Serovar L_2 EBs treated with trypsin (1 mg/ml) or Tris buffer at 37°C for 30 min were allowed to adsorb to HeLa cells coverslip cultures at room temperature for 2 hours. Half the coverslips were treated with 100 units/ml of heparin for 30 min. to elute attached but unendocytosed EBs. All coverslips were washed with PBS and lysed with 2% SDS. Radioactivity of lysates was determined in a scintillation counter.

endocytosis parameters of L_2 EBs. Thus enhancement of infectivity is due to an intracellular event. We therefore turned to biochemical methods to measure possible changes in chlamydial enzymatic activity after outer membrane proteins of EBs were altered.

E. Biochemical Studies

Previous work on studying EB to RB differentiation that showed UTP was incorporated into RNA molecules when EBs were treated with 2-ME in the presence of transcription reaction mix containing nucleotide triphosphates (Sarov and Becker, 1971).

We also considered that ATPase activity may be important in the initiation of EB intracellular differentiation. Hatch et al, (1982) showed the presence of a membrane-bound ATPase in RBs. We therefore measured UTP uptake and ATPase activity in serovar L_2 EBs after treatment with trypsin and 2-mercaptoethanol.

1. Incorporation of UTP

Figure 29 shows the effect of trypsinization on UTP uptake into serovar L_2 EBs. In three separate experiments, increased ^{32}P-UTP incorporation was evident in trypsinized EBs when compared to control EBs.

Figure 30 shows the effect of 2-mercaptoethanol on UTP incorporation of serovar L_2 EBs. EBs not exposed to 2-mercaptoethanol did not exhibit significant levels of

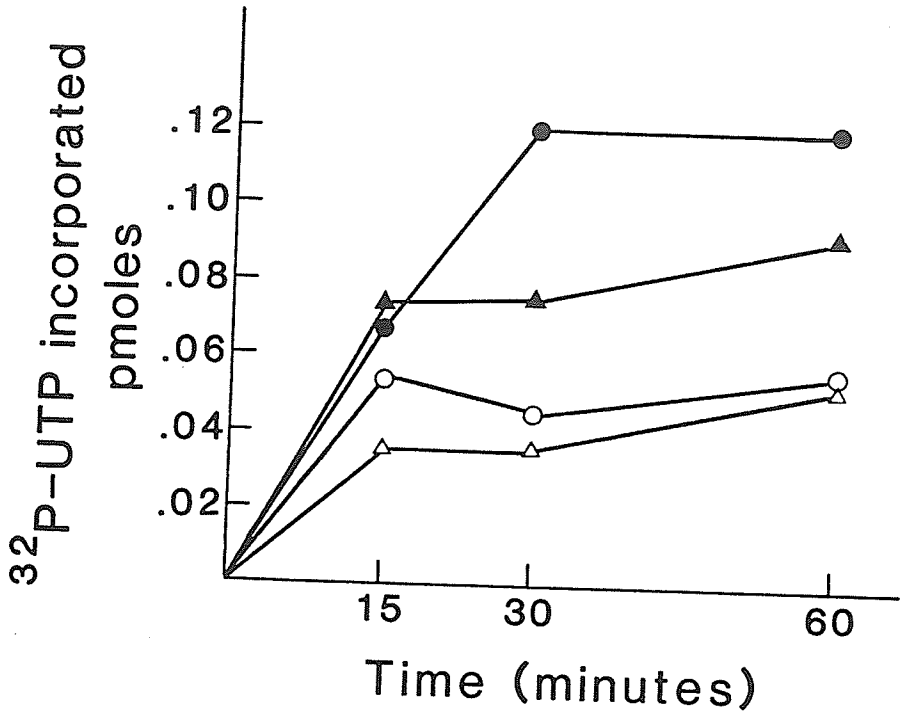


FIGURE 29. Incorporation of ^{32}P-UTP into acid insoluble material by trypsinized or buffer treated C. trachomatis EBs. Serovar L_2 EBs treated with trypsin (●) or tris buffer (▲) were combined with an equal volume of transcription buffer containing ATP, CTP, GTP and ^{32}P-UTP. Controls were prepared similarly with the omission of unlabelled nucleotides for both trypsinized (O) and buffered treated EBs (△). Aliquots were taken at times indicated and precipitated in 10% TCA and 1% sodium pyrophosphate. EBs were filtered, washed with TCA, rinsed in ethanol and counted in POPPOP-toluene when dried.

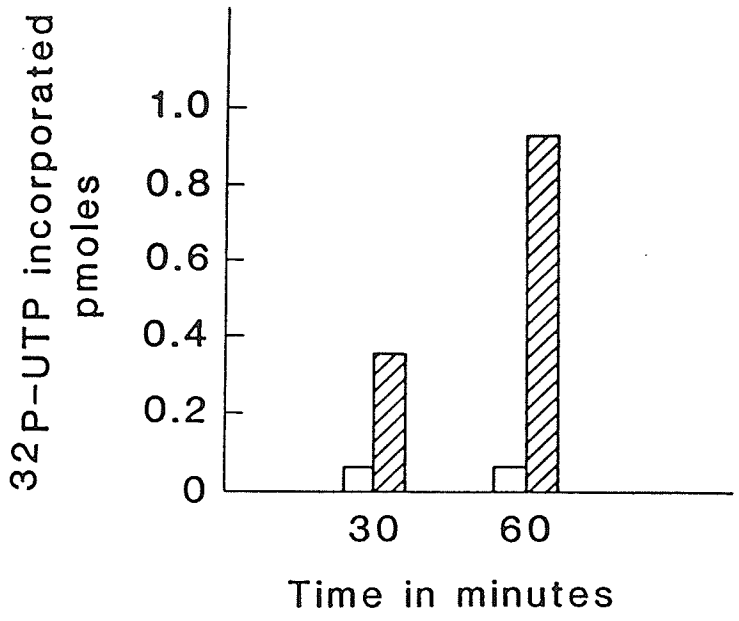


FIGURE 30. Incorporation of ^{32}P-UTP into acid insoluble material by 2-mercaptoethanol treated C. trachomatis EBs. Serovar L_2 EBs were treated with 2 mM 2-mercaptoethanol (hatched bars) or buffer alone (open bars) and combined with an equal volume of transcription buffer. Incorporation of ^{32}P-UTP into acid insoluble material was determined as described for Figure 29

^{32}P-UTP incorporation when incubated with a complete transcription mix at 25°C for an hour. These values are similar to the non-specific levels of incorporation seen in 2-mercaptoethanol treated EBs when nucleotide triphosphates other than ^{32}P-UTP were omitted from the transcription mix. When EBs were treated with 2 mM of 2-mercaptoethanol, the TCA precipitable count of ^{32}P-UTP incorporated increased from the background value of 0.06 p moles to 0.36 p moles at 30' and to 0.93 p moles at 1 hour.

2. High Resolution ^{31}P Nuclear Magnetic Resonance Studies of ATPase Activity

The ratio of the peak intensities of Pi to that of the γ -phosphate peak of the ATP molecule yields a measurement of the ATPase activity seen when ATP is hydrolyzed to ADP and Pi (Figure 31).

Each spectrum represents the accumulation of ATPase activity at 37°C over 12 min from the time the measurement was initiated. This is because biological materials are normally present in specimens at low concentrations. As the amplitude of the NMR signal depends on the number of contributing nuclei, a great number of scans are required to give a significant signal to noise ratio. Four hundred scans were performed in these assays.

FIGURE 31

^{31}P Nuclear Magnetic Resonance Spectrum of ATP

Spectrum shows peak assignments for various phosphate groups of ATP and products of ATP hydrolysis

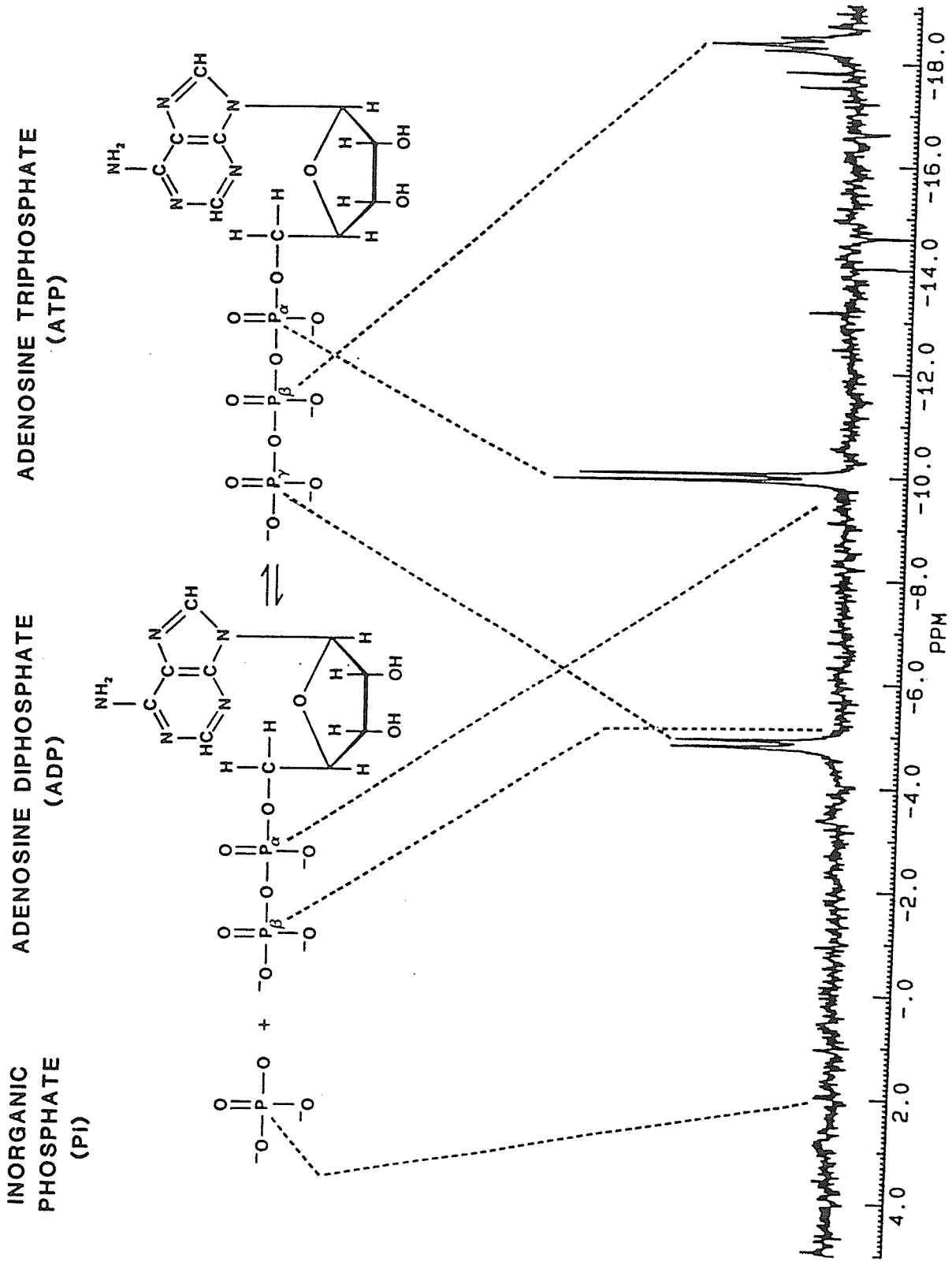

INORGANIC PHOSPHATE (Pi)
ADENOSINE DIPHOSPHATE (ADP)
ADENOSINE TRIPHOSPHATE (ATP)
4.0
2.0
-.0
-2.0
-4.0
-6.0
-8.0
-10.0
-12.0
-14.0
-16.0
-18.0
PPM

Figure 32 shows the ATPase activity and ^{31}P NMR spectra of trypsinized L_2 EBs compared to untreated EBs. EBs were treated with 1 mg/ml of trypsin for 30 minutes, centrifuged and resuspended in a Tris buffer containing 2 mM ATP. The NMR measurements were made at 37°C over three hours. After 3 hours of incubation with 2 mM ATP, the trypsinized EBs show an increase in ATPase activity which was approximately twice that of control EBs.

Figure 33 shows the effect of 2-mercaptoethanol on ATPase activity of serovar L_2 EBs measured by NMR spectroscopy. There was significant ATPase activity among EBs exposed to 2-mercaptoethanol. This was initially present at maximal levels and no further increases occurred over an hour. Perhaps the concentration of ATP was rate limiting. The ATPase activity was magnesium ion dependent as no Pi peak was observed when magnesium salts were omitted.

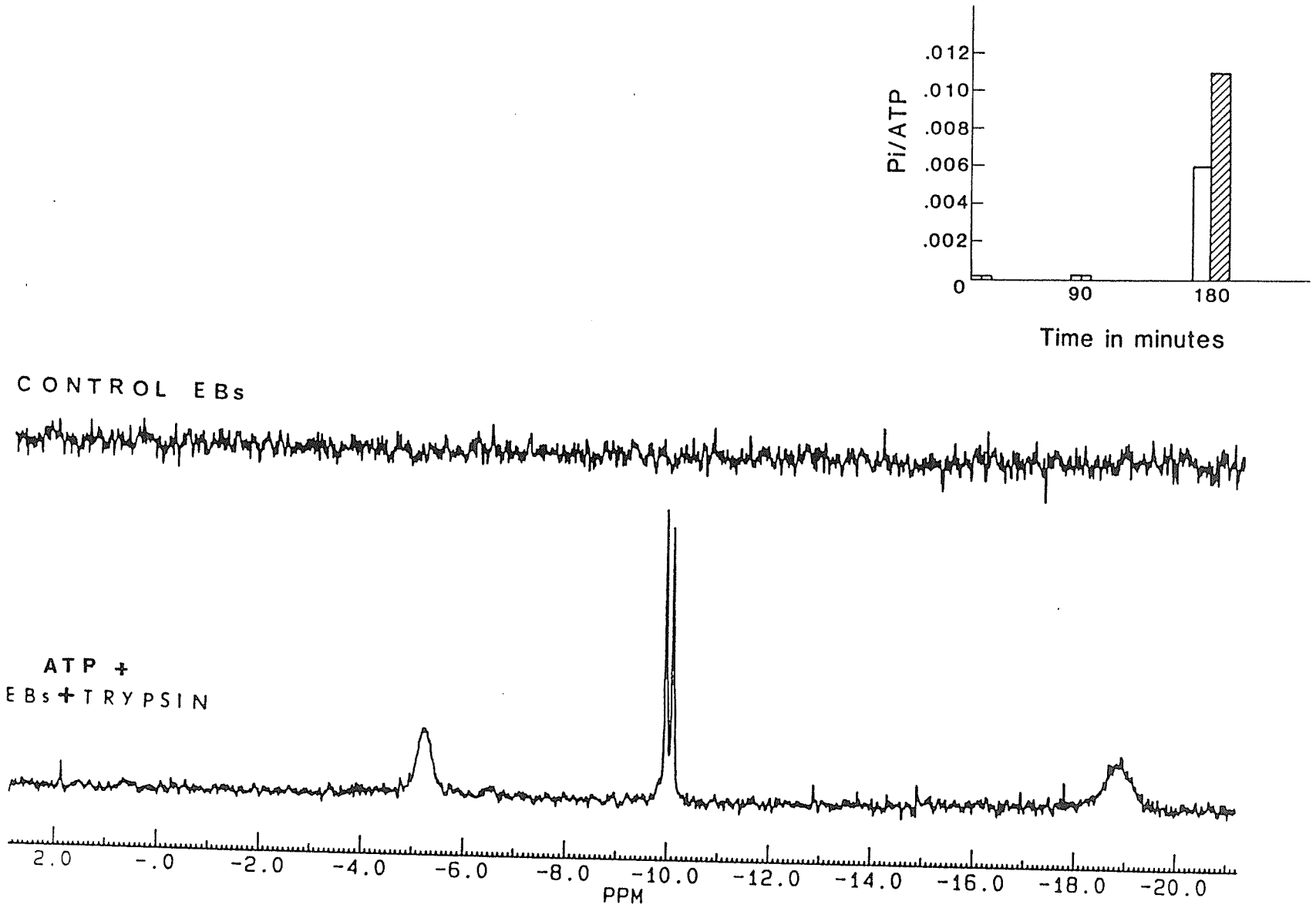
These results suggest that trypsinized EBs may be favoured for intracellular survival by virtue of having increased membrane permeability to nucelotides essential for early differentiation and early gene transcription, perhaps through limited cleavage of MOMP, the major pore forming protein of the organism. Furthermore, the results observed with 2-mercaptoethanol confirm the important role of reduction of the outer membrane in promoting biochemical events characteristic of RB development. These results also suggested a way to investigate a mechanistic explanation for the

FIGURE 32

^{31}P Nuclear Magnetic Resonance Spectra of

Control and Trypsinized EBs

Serovar L_2 EBs were treated with 1 mg/ml of trypsin or buffer alone at $37^{o}C$ for 15'. EBs were pelleted and resuspended in ATP buffer containing 2 mM ATP. ATPase activity was monitored in a Bruker AM-300 NMR spectrometer. Inset shows ATPase activity as measured by the ratio of Pi/ATP in control (open bars) and trypsinized EBs (hatched bars)


.012
.010
.008
.006
.004
.002
0
Pi/ATP
90
180
Time in minutes
CONTROL EBs
ATP +
EBs + TRYPSIN
2.0
-.0
-2.0
-4.0
-6.0
-8.0
-10.0
-12.0
-14.0
-16.0
-18.0
-20.0
PPM

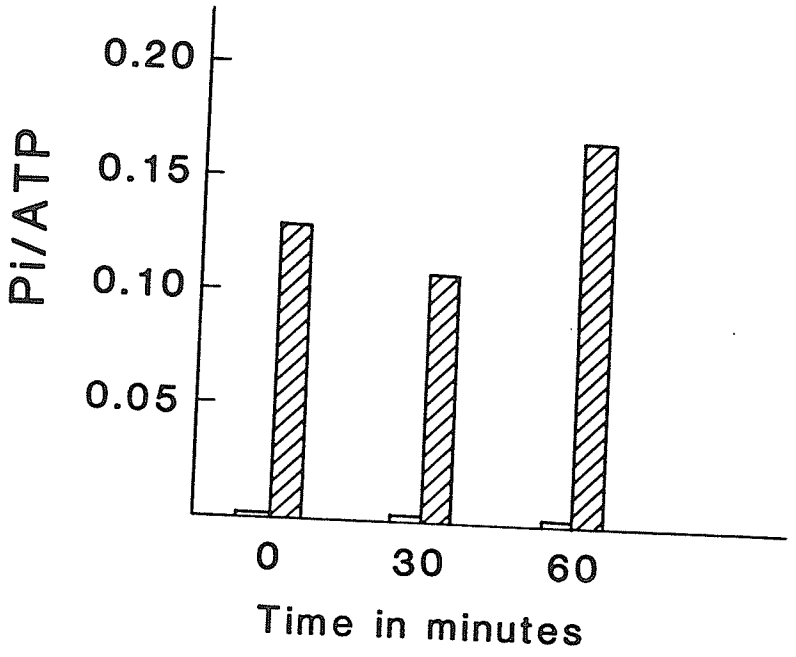


FIGURE 33. ATPase activity in 2-Mercaptoethanol treated EBs of C. trachomatis. Serovar L_2 EBs were treated with 2 mM 2-ME (hatched bars) or buffer alone (open bars) and then combined with an equal volume of ATP buffer containing 2 mM ATP. ATPase activity was determined as the ratio of intensities of the Pi peak to that of the γ -phosphate of ATP.

intracellular neutralization of C. trachomatis EBs observed with anti-MOMP Mabs. We reasoned that neutralizing Mabs may be able to cross-link MOMP and prevent the increase in membrane permeability.

3. Inhibition of UTP incorporation and ATPase activity of 2-mercaptoethanol treated EBs

To perform inhibition studies on 2-mercaptoethanol treated EB using a neutralizing Mab, it was necessary to ascertain that the 2-mercaptoethanol concentration used does not affect the reaction by reducing the antibody molecule. Figure 34 shows that at the concentration (2mM) of 2-mercaptoethanol used in the UTP uptake and ATPase experiments neutralization reactions were not adversely affected.

Table 14 shows that antibody treated EBs exposed to 2-mercaptoethanol exhibited a decrease in ^{32}P-UTP incorporation when compared with control reduced EBs. The reduction of ^{32}P-UTP incorporation was greatest when lower concentrations of EBs were used. ^{32}P-UTP incorporation was reduced by 61% when 10^7 EBs were used, compared to 19% when 10^8 EBs were used. Similarly, Fig. 35 shows ^{31}P NMR spectra of ATPase activity in 2-ME treated EBs pre-incubated with monoclonal antibodies. No Pi peak was observed for EBs treated with neutralizing Mab UM-4. The LPS Mab which does not neutralize serovar L_2 infectivity exhibited a Pi/ATP ratio of 0.0504 compared to 0.0991 for EBs incubated with normal mouse IgG.

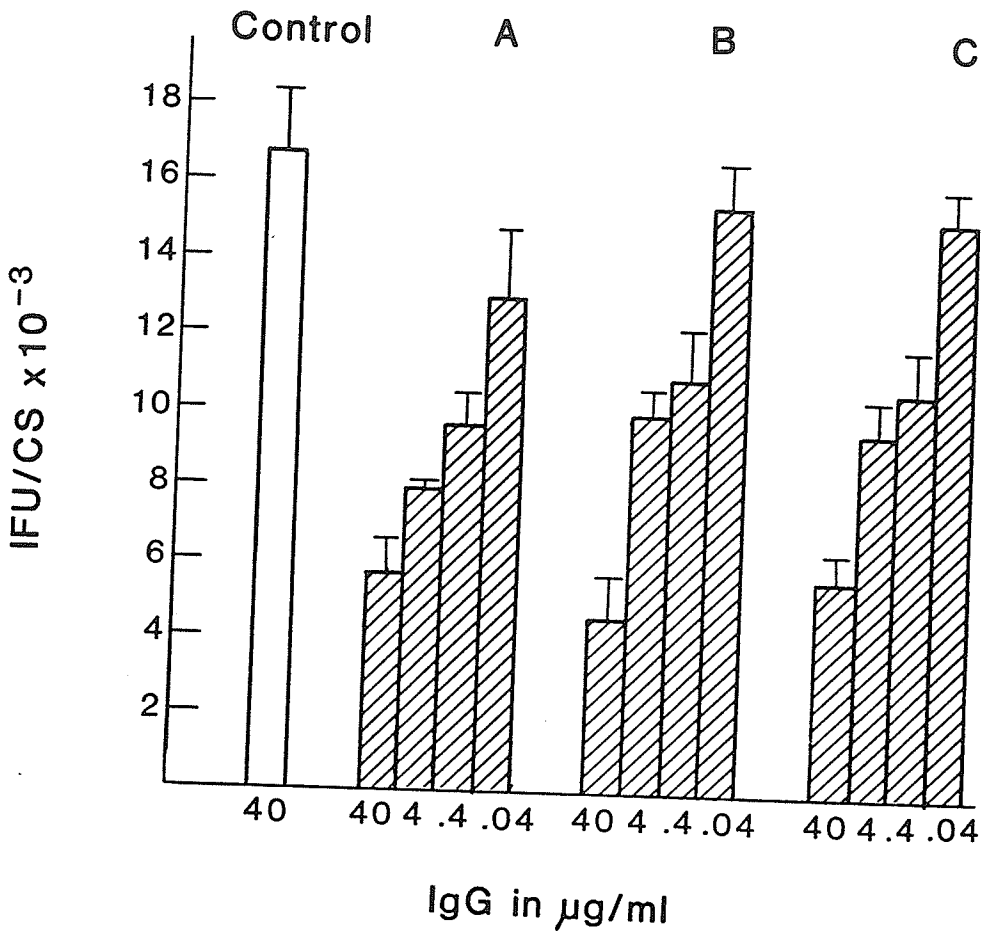


FIGURE 34. Effect of 2-mercaptoethanol on the neutralization of infectivity of C. trachomatis EBs. Purified L_2 EBs were incubated with normal mouse or UM-4 IgG at 37°C for 30 min in the absence of 2 mM 2-Mercaptoethanol (2-ME) (A). In (B), EBs were pre-treated with 2-ME for 30 min before being incubated with antibody. In (C), EBs were incubated with antibody at 37°C for 30 min before being treated with 2 mM 2-ME. Residual activity for HeLa 299 cells were determined by counting IFUs per coverslip culture.

TABLE 14

EFFECT OF NEUTRALIZING MAB ON UTP UPTAKE INTO 2-ME TREATED EBs

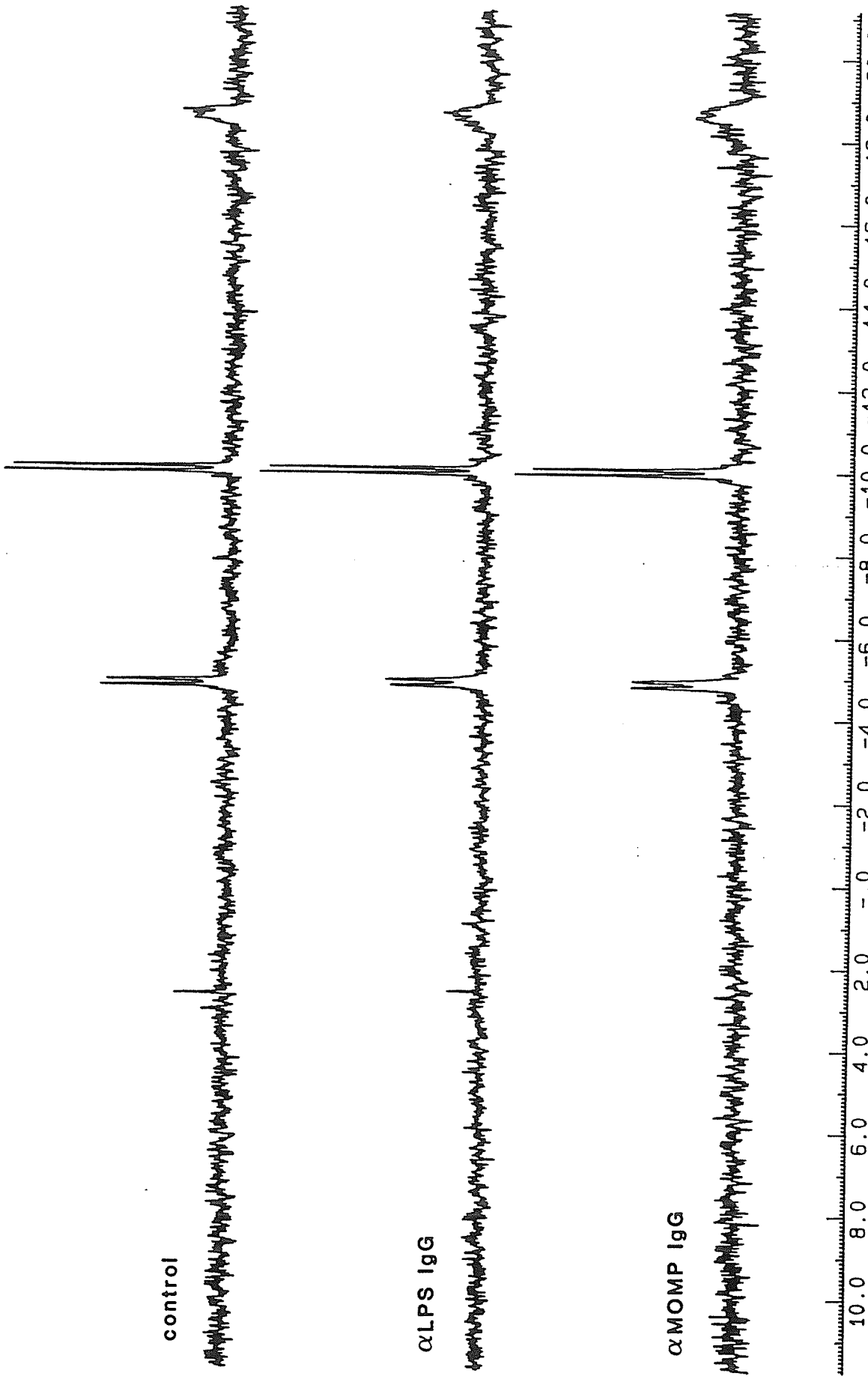
	UTP (p moles) Incorporated		
No. of EBs	Control	Mab	% Reduction
10^7	0.30	0.12	61%
5×10^7	0.57	0.42	26%
10^8	0.78	0.63	19%

Serovar L_2 EBs were incubated with IgG from Mab UM-4 or normal mouse serum at 37°C for 30 min. The mixtures were then combined with an equal volume of transcription mix containing ^{32}P-UTP and 2 mM 2-ME. The incorporation of ^{32}P-UTP into TCA precipitable material by EBs in each sample at 25°C for 60 min was determined by scintillation counting. Percentage reduction was calculated as the % reduction in cpm for Mab-treated EBs compared to control EBs

FIGURE 35

^{31}P Nuclear Magnetic Resonance Spectra Showing ATPase Activity in Antibody-treated EBs of C. trachomatis

Serovar L_2 EBs were incubated with IgG from normal mouse serum, anti-LPS Mab (UM-7) or anti-MOMP Mab (UM-4) at 37°C for 30'. The reaction mixtures were then added to ATP buffer containing 2 mM 2-ME and 2 mM ATP. ATPase activity was monitored in a Bruker AM-300 NMR spectrometer.


control
αLPS IgG
αMOMP IgG
10.0
8.0
6.0
4.0
2.0
-.0
-2.0
-4.0
-6.0
-8.0
-10.0
-12.0
-14.0
-16.0
-18.0
-20.0
PPM

VI. DISCUSSION

The goal of my work has been to define the molecular pathogenesis of eucaryotic cell infection exploited by C. trachomatis. My hypothesis is that outer membrane proteins of C. trachomatis determine the early events of attachment, endocytosis and intracellular differentiation. Special adaptations of outer membrane proteins underlie the successful parasitism of C. trachomatis.

Three approaches were used to examine the role of outer membrane proteins in pathogenesis. First, the life cycle of C. trachomatis was examined by transmission and scanning electron microscopy to provide ultrastructural information on the interaction of C. trachomatis with HeLa cells. Secondly, monoclonal antibodies to outer membrane macromolecules were used to study neutralization of chlamydial infectivity. Finally, structural alterations of outer membrane proteins with trypsin and 2-mercaptoethanol were studied to determine biochemical changes that correlate with infectivity.

The ultrastructural studies of the infection with C. trachomatis of HeLa cells was useful in defining the early events in the pathogenesis in eucaryotic cells. Two interesting observations from these electron micrographs pertaining to the role of the outer membrane in infection are i) EBs were not endocytosed via clathrin-coated pits; ii) outer membrane of replicating RBs appeared to be contiguous with the inclusion membrane.

The mechanism of endocytosis of EBs into eucaryotic cells is still an unresolved issue. For HeLa cells, the size of the coated pits that we observed were mostly only 100 nm in diameter and were not used by C. trachomatis EBs to enter the cell. In contrast, Wyrick et al (1986) has electron microscopy evidence that C. psittaci are internalized into mouse L cells via clathrin-coated pits. As described in the literature review, there are also biochemical studies which support either mechanism of endocytosis. The two mechanisms may not be mutually exclusive.

The other interesting observation from our electron micrographs related to the chlamydial outer membrane is the apparent fusion between the RB outer membrane and the inclusion membrane when RBs marginate the inclusions. Using freeze-replica imaging, Matsumoto (1981) observed surface projections on RBs being connected to the inclusion membrane. These observations are helpful when we consider our hypothesis on the porin activity of the reduced or trypsinized membrane. Somehow, host cell nucleotides and other precursor molecules must cross the inclusion membrane so that they are available to be taken up by chlamydiae. If the inclusion is an invagination of the host plasma membrane, it will be impermeable to the nucleotides unless it was modified. The fusion of the RB membrane with the inclusion membrane to form porin channels is a possible solution. Ziechner isolated macrophage inclusion membrane containing C. psittaci and found that

of approximately 10 proteins resolved on SDS-PAGE, only two were simultaneously found in host plasma membrane. Thus extensive modification of the vacuolar membrane possibly by insertion of RB outer membrane may facilitate influx of host nutrient pools into RBs.

While ultrastructural information is helpful, structure-function relationships can be better probed with monoclonal antibodies.

Our second approach was to make a library of monoclonal antibodies using whole native EBs. Ninety-nine hybridomas were examined for neutralization of C. trachomatis infectivity in the HeLa 229 cells. Although we had previously published a neutralization paper establishing that a Mab to an epitope MOMP can neutralize infectivity in vitro (Peeling, et al., 1984), other researchers have found varying or limited success with an in vitro neutralizing assay. Zhang et al (1986) found Mabs that were protective in mice and in the monkey-eye model and yet could not demonstrate in vitro neutralization activity for these Mabs. Kuo et al (1985) found no neutralization with any Mab without complement. We believe that an in vitro assay of neutralization of infectivity is a relevant method to understand chlamydial pathogenesis. Structure-function relationships can be deduced from the molecular specificity of Mabs and their mechanism of neutralization. Animal models are expensive, often unavailable (as in the case of monkeys), and are more

complex in terms of defining stages of the chlamydial life cycle at which inhibition takes place.

We examined the parameters of the neutralization assay that may influence the effectiveness of the assay. We used the neutralization assay previously published as a basis to examine the individual components of the reaction, i.e. the antigen, the antibody and the reaction conditions.

The antigen used in this assay was the native EB. Our first concern was that the EB is heat labile over the incubation period used for the neutralization reaction. Our results and reports in the literature showed that thermal inactivation of EBs in the absence of protein can amount to 50% or more of the inoculum in an hour. Non-viable EBs may affect the neutralization assay by binding antibody and decreasing its effective concentration. Our results suggest that the reaction mix should contain at least 10 ug/ml immunoglobulin protein to maintain the viability of the EBs.

The thermal stability of EBs in the presence of 10 ug/ml of NMIgG or bovine serum albumin (BSA) was compared to phosphate buffered saline. NMIgG was the best stabilizing agent. The exact mechanism of this stabilization is not known at present. Since NMIgG was our antibody control in these reactions, the Mab antibody dilutions were subsequently always made in 10 ug/ml of NMIgG to ensure that there is no unnecesary heat inactivation of the antigen.

Our next concern was that EBs are normally suspended in an hypertonic salt solution called SPG, containing about 7.5% sucrose, phosphate, and glutamate. Although it is superior to PBS in preserving EB viability, the high salt concentration may decrease the effectiveness of the antigen-antibody reaction especially if the reaction is dominated by ionic interactions (Paul, 1984). Figure 21 shows the comparative effectiveness of using SPG or PBS as antigen diluent. SPG is clearly the better diluent. It probably speaks for the reaction being largely non-ionic in nature.

As for the antigen-antibody reaction, the basic principles of a bimolecular interaction apply. Provided antibody does not alter the antigen on binding, the reaction is reversible:

$$Ag + Ab \rightleftharpoons Ag\text{-}Ab$$

The thermodynamics and kinetics of these reactions obey the law of mass action:

$$Ka = \frac{[AG\text{-}Ab]}{[Ag]\,[Ab]}$$

Where Ka = affinity constant

[Ag] = concentration of antigen

[Ab] = concentration of antibody

[AgAb] = concentration of the antigen-antibody complex

Thus, the ratio of the concentrations of the antigen-antibody complex to the reactants should always be at equilibrium. Provided neither reactant concentrations are limiting, changing the concentration of either antigen or antibody will change the concentration of the complex. The antigen concentration is limited by the size of the coverslip used for monolayer cultures. The ideal limits of reliable inclusion counting are between 10^3-10^4 inclusions forming units per coverslip although one log variation either way can be accommodated.

As is typical of reactions involving a bivalent antibody and a multi-valent antigen in solution, there is a zone of equivalence where antigen and antibody concentrations are optimal for binding. We had previously shown a prozone phenomenon in the presence of antibody excess (Peeling et al, 1984) and Figure 22 again shows the decreased effectiveness of neutralization infectivity at excessive concentrations of antibody. This observation is consistent with the necessity for bivalent binding of the antibody to the EBs for effective neutralization.

These observations also give an initial clue as to the mechanism of neutralization of chlamydial infectivity with anti-MOMP antibodies. Neutralization may not simply be masking an essential surface molecule but bivalent binding of antibody resulting in cross-linking of MOMP epitopes may be the physical basis for neutralization. The cross-linking hypothesis is

supported by Caldwell and Perry (1982) who showed that intact IgG is required for neutralization of MOMP as Fab fragments alone were not sufficient for neutralization.

One important corollary of the mass action law is that since it is concentration and not absolute amounts of the antigen or antibody that enter the equation, putting the reactants in a smaller volume will favour the amount of complex formed. These differences vary approximately as the square of the volume, (Paul, 1984) We compared our reaction volume of 360 ul to 1 ml used by other investigators and found a corresponding decrease in effectiveness of neutralization by the same Mabs as the volume of the reaction increases. If 50% neutralization of infectivity is chosen as the arbitrary standard for labelling an antibody as neutralizing, then Mabs UM-4 which is usually > 90% neutralizing becomes only borderline positive in a 1 ml reaction volume.

Other important factors to consider are the temperature and length of incubation of the reaction mix. For any antigen-antibody interaction, the second law of thermodynamics states that:

$$\Delta G^{o} = \Delta H^{o} - T\,\Delta S^{o}$$

ΔG^{o} = standard free energy change

ΔH = Change in enthalpy (heat of reaction)

ΔS = entropy (change in disorder)

T = temperature

The forward reaction of antigen-antibody association is not temperature dependent. However, raising the temperature thermodynamically favours complex formation through increased diffusion of reactants but also kinetically favours complex dissociation through increasing entropy. We compared the effectiveness of neutralization at 4°C for one hour to 37°C for 30 minutes. The results showed that even with constant agitation, there was very little neutralizing activity at 4°C for 1 hour using the same Mab that neutralize very effectively at 37°C for 30 minutes.

In their excellent experiments examining antibody interactions with membrane antigens, Mason and Williams (1980) concluded that although the rate of complex association increases two-fold when the temperature was raised from 4°C to 23°C, the rate of complex dissociation increase more than ten-fold over the same temperature range. As a result, the binding reaction should be favoured at low temperature. However, though this theory is sound for univalent binding of molecules in solution or antigens on a cell membrane, they observed that dissociation is much slower for bivalent binding. Our results may possibly be explained by our hypothesis that neutralizing antibodies bind bivalently, i.e. must cross-link MOMP epitopes, in order to be effective in neutralization.

These results indicate that the optimal conditions for the neutralization assay are to incubate the

reaction mixture at 37°C for 30 minutes. The antigen concentration should yield about 10^3-10^4 IFUs/coverslip. The volume of the reaction should be as small as possible to increase the efficiency of the reaction. This information may also explain some of the variability of neutralization tests seen by other experimenters. Without stating these parameters of the neutralization assay, an arbitrary 50% endpoint irrespective of antigen and antibody concentration or conditions of incubation can be meaningless.

Using an optimal neutralization assay, we found that three Mabs to L_2 MOMP were able to neutralize infectivity. We also observed that for Mabs to MOMP, the extent of neutralization was correlated to the intensity of binding as shown by the dot-blot assay (Figure 17). Previous work with whole serum or eye secretions showed that neutralization was type-specific (Barenfanger and MacDonald, 1974; Howard, 1975). With monoclonal antibodies to MOMP, Lucerno and Kuo (1985) found that 5 of 7 of their type specific Mabs, and two of five of their subspecies-specific Mabs neutralized infectivity. We also found that our type-specific L_2 Mab, UM-4 was neutralizing for serovar L_2. From the secondary structure predictions of MOMP, based on the primary amino acid sequence, it appears that the type-specific determinant is in a prominent hydrophilic region flanked by two disulphide linked sequences. The prominent surface exposure may explain its immunogenicity and immunoaccessibility. In the dot-blot

assay, UM-4 IgG bound very strongly to L_2. This reflects high binding avidity of the Mab and immunoaccessibility of the epitope on native EBs.

Of our four subspecies MOMP antibodies, UM-1 neutralized serovar L_2 infectivity, UM-6 neutralized serovar A while UM-2 neutralized serovar G. Again, these results correlated well with the intensity of binding in dot-blot assay. UM-3 and UM-5 did not bind to any of the native EBs and did not neutralize infectivity for any EBs. UM-1 neutralized serovar L_2 but not B or D. This was reflected in their binding pattern in the dot-blot assay where the binding of UM-1 to L_2 was stronger than that of B and D. C11 which neutralize only L_2 EBs bound with equal intensity to serovar L_2, F, and G EBs. This apparent lack of correlation between neutralization and immunoaccessibility is the only exception we have observed.

We attempted to correlate in vitro neutralization with protection. The best animal model for protection studies is the monkey eye model. However, as it is unavailable and Zhang et al (1986) found that the mouse toxicity prevention test (MTPT) showed an excellent correlation with the monkey eye protection, we therefore evaluated one Mab by both in vitro neutralization and MTPT. By in vitro assay on HeLa cells, Mab, UM-1 neutralized serovar L_2 but not serovar D. The results with the MTPT were also consistent with the neutralization assay. Due to the expense of the MTPT,

we did not test other Mabs. Zhang et al found that protective Mabs by the MTPT and the monkey eye model were correlated with immunoaccessibility of the epitope on native EBs. But they failed to show in vitro neutralization with any of the protective Mabs using HeLa cells. We are not certain if their experimental conditions for neutralization were entirely comparable with ours.

The mechanism of neutralization of infectivity may be the result of inhibition of attachment or endocytosis or inhibition of intracellular development. There were no significant differences in the rate or efficiency of antibody-treated EB attachment or uptake to host cells compared to control EBs treated with NMIgG when anti-MOMP Mabs were studied. This is contrary to the early work by Byrne and Moulder (1978) and Ainsworth et al, (1979) which showed that antibody coated EBs were not taken up as much as native EBs. They did not distinguish between the stages of attachment and endocytosis. Since whole serum was used, there might well have been antibodies that inhibited attachment or endocytosis in it. Our results are consistent with those of Caldwell and Perry who used polyclonal monospecific anti-MOMP antibodies (1982).

The inhibition of phagolysosomal fusion is the first intracellular event that may be affected by antibody-treated EBs. Early work by Friis (1972) suggested that neutralization of antibody-treated EBs may be due to plasma membrane enzyme activation during

formation of the phagosome. Since inhibition of phagolysosomal fusion appeared to be specified by the outer membrane of EBs (Eissenberg et al, 1985), it is entirely possible that coating EB with antibody can prevent it from performing that function. However, when we examined coverslip cultures inoculated with the neutralization reaction mix and stained with fluorescein conjugated anti-mouse IgG, antibody-treated EBs remained in the cell 20 hours post-infection at a time when control organisms have differentiated into RBs. We cannot discount the possibility that some neutralized EBs may be destroyed by phagolysosomal fusion, but the persistence of neutralized organisms as EBs is most consistent with an effect of antibody on the reorganization of EBs to RBs.

Our hypothesis of the intracellular mechanism for neutralization of infectivity was deduced from our results and that of others. Caldwell and Perry (1982) found that monovalent Fab fragments of the neutralizing anti-mouse IgG failed to neutralize infectivity even though they were shown to have bound the EBs. The presence of a prozone phenomenon at high concentrations of antibody is also consistent with bivalent antibody binding being necesary for effective neutralization. By cross-linking MOMP epitopes, antibody probably contributes to the rigidity of the outer membrane so that EB to RB reorganization cannot take place. Anti-MOMP antibodies may also alter outer membrane permeability such that the EB remains

impermeable to nutrients and precursor molecules for biosynthesis and thus remain in the host cell apparently unchanged as visualized by fluorescein staining at 20 hours post-infection.

Our discussion on the mechanism of antibody mediated neutralization of infectivity ends here with the conclusion that our fluorescein staining work showed that neutralization by Mabs to MOMP appears to be an intracellular event and that antibody-treated EBs appear to be "frozen" in the EB stage while normal ones proceed to become RBs. The ideas on the mechanism of neutralization will be further developed after the discussion of results from the work on alteration of the EB outer membrane.

Since some of the early event of C. trachomatis eucaryotic cell infection are specified by OMPs, we reasoned that alteration of structure may allow detection of altered function. As discussed in the literature review section, the differentiation of EBs to RBs involves a change in size approximately from 300 nm to 1000 nm and reduction of disulphide linkages in the outer membrane. RBs become permeable to nutrients and precursor molecules for biosynthesis. Eissenberg et al, (1983) observed release of C. psittaci EB envelope material in peritoneal macrophages and L cells in the absence of phagolysosomal fusion. This is consistent with the belief that spontaneous breakdown or autolytic enzyme release of EBs envelope components occurs prior to the differentiation of EBs to RBs. Thus, we altered

the outer membrane of EBs using trypsin and 2-Mercaptoethanol and tried to correlate the resulting structural changes with changes in metabolic activity.

Hackstadt and Caldwell (1985) found that trypsinized EBs had unaltered infectivity. The investigators reported the results for a single concentration of trypsin (50 ug/ml) and assayed infectivity at a single time after exposure (two hours). When we used different trypsinization conditions, we found strikingly different results.

Short duration of exposure (< 1 hour) enhanced infectivity by more than two-fold. Longer duration of exposure (≥ 4 hours) greatly reduced infectivity. A dose response effect was apparent with the highest concentration of trypsin maximally enhancing and inhibiting chlamydial infectivity. Trypsin enhancement was seen with two serovars of L_2 and a D representative of the two biovars of C. trachomatis suggesting it is a general phenomenon of C. trachomatis.

We correlated enhancement of infectivity with proteolysis other proteins of chlamydial membranes. ^{125}I radiolabelling of the outer membrane showed that MOMP and a 75 kDa protein underwent most changes in protein structure and antigenicity. The major cleavage products of MOMP were retained on the surface of trypsinized EBs in cleaved form as 3 fragments.

We next considered the mechanism of enhancement of trypsinization. Both untreated and treated trypsinized EBs were unaggregated as determined by electron

microscopy. Trypsinized EBs also did not exhibit enhanced HeLa cell binding or endocytosis. We can thus conclude that the enhancment of infectivity by trypsin is occurring at a step after cell entry. Two possible intracellular mechanisms may be considered: 1) membrane protein changes may be required for EB to reticulate body (RB) reorganization, or 2) to assist EBs in evading endosomal fusion with host cell lysosomes. We consider the former mechanism more likely. Pearce et al. (1986) previously reported that only 12% of endocytosed serovar L_2 EBs underwent lysosomal fusion. Therefore, augmentation of avoidance of lysosome fusion could not account for the more than two-fold enhancement of infectivity exhibited by trypsinized EBs.

We consider that the mechanism of enhancement is associated with EB to RB reorganization and may be associated with a change in outer membrane permeability. We used two methods for detecting increased permeability of the outer membrane. We reasoned that since chlamydiae are energy parasites, the first intracellular requirement must be the acquisition of ATP. Chlamydia RBs are known to possess an ATP-ADP translocase that will transport host ATP in exchange for ADP. Reticulate bodies also possess an ATPase that will reduce ATP to ADP and Pi (inorganic phosphate) and in doing so, use the energy of the reaction for lysine import (Hatch et al, 1982). In hydrolysing ATP, the RBs establish an energized membrane for uptake of nutrients. Our

hypothesis is that the EB also possesses intrinsic ATPase activity but that it is restricted by the impermeability of its outer membrane. Hatch et al (1986) were unable to detect ATPase in C. psittaci EBs. However, they used triton X-100 in their EB purification process which might have adversely affected the EBs. They used thin-layer chromatography to monitor hydrolysis of ATP. High resolution ^{31}P NMR spectroscopy may be better suited to detect ATP hydrolysis since the appearance of Pi in the reaction mix can be easily monitored. No radioactive materials are required and the whole reaction can be continuously monitored at the desired temperature for any length of time. The intensity of the signal (measured from the integral of the area under the peak) is proportional to the number of nuclei that contribute toward it. The absolute amount of Pi cannot be accurately measured because of the insensitivity of NMR spectroscopy for biological materials which are usually present in very low concentrations. Valid comparisons are made by comparing the ratio of peak intensity of Pi to that of the γ-phosphate of ATP.

RNA polymerase activity can also be used as a measure of increased outer membrane permeability. Sarov and Becker (1971) showed that C. trachomatis possesses a pre-existing DNA-dependent RNA polymerase. Using ^{32}P-UTP, we looked for TCA precipitable ^{32}P-UTP incorporation into trypsinized EBs. Trypsinized EBs consistently showed more RNA polymerase activity than

untreated EBs in three separate experiments. Native EBs are impermeable to nutrients and nucleotide triphosphates and it is quite possible that in vitro trypsinization can make the outer membrane more permeable.

By ^{31}P NMR spectroscopy, we did not find detectable pre-existing pools of ADP, ATP or Pi in native EBs. When EBs were trypsinized and incubated in the presence of 2 mM ATP over 3 hours, trypsinized EBs showed twice the amount of ATPase activity as did untreated EBs. This activity appears to be magnesium dependent. Hatch had reported similar magnesium dependence for RB ATPase activity. The EBs we used had been extensively purified and filtered to exclude any contaminating RBs. The ATP buffer was monitored for 6 hours at 37°C and did not spontaneously break down during the course of the experiment.

Thus we conclude here that protease "activation" of EBs whether of endogenous or exogenous origin may assist intracellular differentiation of C. trachomatis by enhancing the outer membrane permeability to nucleotides.

EBs treated with 2 mM of 2-mercaptoethanol showed 10-15 times the RNA polymerase activity compared with untreated EBs. ^{31}P NMR measurements showed that within 10 minutes of incubation with ATP at 37°C, magnesium dependent ATPase activity was evident in 2-mercaptoethanol treated EBs.

Taken together, these results are consistent with enhanced permeability of the outer membrane of EBs, as evidenced by the uptake of nucleotide triphosphates, using 2-mercaptoethanol and trypsin. These results are also consistent with presence of a pre-formed ATPase enzyme complex in EBs. Hatch et al (1986) failed to stimulate metabolic activities in EBs of C. trachomatis and C. psittaci after they were reduced with dithiothreitol. However, their EBs were harvested from the lysis of host cells by Nonidet P-40, a nonionic detergent. While the detergent may aid the purification of EBs from contaminating RBs, the EBs may be adversely affected as well.

The major effect of trypsin on EBs is the degradation of MOMP. Similarly, reduction of disulphide linkages in the outer membrane by 2-mercaptoethanol may affect MOMP preferentially since it is the major cysteine rich outer membrane protein. Thus the biologic and biochemical effects observed with trypsin and 2-mercaptoethanol may be due to effects on MOMP.

Bavoil et al (1984) first showed porin activity in the outer membrane of EBs by a liposome swelling assay when they reduced the EBs with dithiothreitol and blocked the free sulfhydroxyl groups with iodoacetamide. They suggested but did not prove that MOMP was the major porin protein in their assay.

When the properties of other bacterial porins are compared with MOMP, there are numerous similarities.

Most bacterial porins possess a molecular mass in the range of 30-40 kDa. That of MOMP is about 39 kDa. Bacterial porin usually occur in trimers and are unusually rich in β-sheet structures containing 11-15 amino acid residues. There are few charged residues and no detectable -helical sequences. The -sheets are perpendicular to the membrane and probably form the water filled channel with hydrophilic amino acids. The amino acid sequence of MOMP has been derived from the nucleotide sequence published by Stephens et al (1986). It has a relatively low content of hydrophobic amino acids. It also lacks helical turns, which are usually associated with sequences having a tetrapeptide, flanked by two hydrophobic amino acid residues. Like most porins, MOMP has an acidic isoelectric point of about 5 (Bavoil et al 1984). Bavoil et al also calculated the pore radius of the reduced outer membrane complex to be 0.65-0.90 nm which corresponds to an exclusion limit range of 850-2,250 daltons. This pore size is sufficient to allow entry of most precursor molecules such as nucleotide triphosphates and amino acids. Other bacterial porins are usually in the range of 1.1-2 nm in pore radius.

To further evaluate the possibility of enhanced UTP uptake and ATPase activity seen with reduced EBs being attributable to enhanced permeation of nucleotides through MOMP, we reacted EBs with neutralizing MOMP Mab and looked for evidence of metabolic activity. We first ascertained that the concentration of 2-mercaptoethanol

was not detrimental to mouse IgG in the course of experiment. The neutralization of serovar L_2 by Mab, UM-4, was identical with and without the presence of 2-mercaptoethanol. Neutralization was equally effective whether 2-mercaptoethanol exposure of EBs occurred before or after antibody binding. 2-mercaptoethanol probably does not affect the antigenic properties of the type-specific region of MOMP even though disulphide bonds are reduced.

No ATPase activity was detected in EBs treated with neutralizing MOMP Mab IgG. Although the Mab to LPS did not neutralize infectivity, it reduced ATPase activity by about 50%. This is probably because LPS molecules are usually found in close proximity to porin proteins (Naikado et al, 1985) and this may represent steric hindrance when the antibody binds.

UTP incorporation was also inhibited by pre-treatment of EBs with neutralizing Mab. The inhibition was a graded response depending on the number of EBs used in the assay. It is very unlikely that the neutralizing Mab specifically inhibits both ATPase and RNA polymerase enzyme activity directly. The reduced incorporation of ^{32}P labelled UTP into antibody-coated EBs is most consistent with the reduction of porin function through MOMP.

Based on the prozone effect we observed and the previous work of Caldwell and Perry (1982) with Fab fragments inhibition of infectivity and reduction of

porin activity likely involves cross-linking of MOMP molecules.

Can an IgG molecule physically cross-link MOMP? The calculations from electron microscopy and computer imaging studies of Chang et al. (1982) showed that MOMP molecules exist as hexagonal arrays with 6 monomer units surrounding a depression 10 nm in diameter. The array is approximately 15 nm across its axis. A mouse IgG molecule with its Fab arms at an angle of 125^{o} can extend to cover a distance of 14.5 nm diameter. Thus, it is physically possible for the MOMP molecules to be cross-linked by bivalent binding of the IgG molecule.

The results from these biochemical studies have been synthesized to construct a hypothetical schematic representation of what may be involved in EB to RB transformation (Figure 36). When MOMP is reduced by 2-mercaptoethanol or cleaved by trypsin, ATP diffuses more freely through the outer membrane to the cytoplasmic membrane. There ATP is taken inside the EB by an ADP-ATP translocase mechanism. Subsequent hydrolysis of ATP by an ATPase enzyme complex, possibly located in the cytosolic side of the cytoplasmic membrane, energizes the inner membrane by the generation of a proton gradient. Uptake of nutrients into the developing EB is then coupled to the electrochemical gradient. In RBs of C. psittaci, Hatch et al. (1982) has documented the presence of an ADP-ATP translocase and that the cytoplasmic membrane is energized when ATP is hydrolysed. We reasoned that EBs should also possess

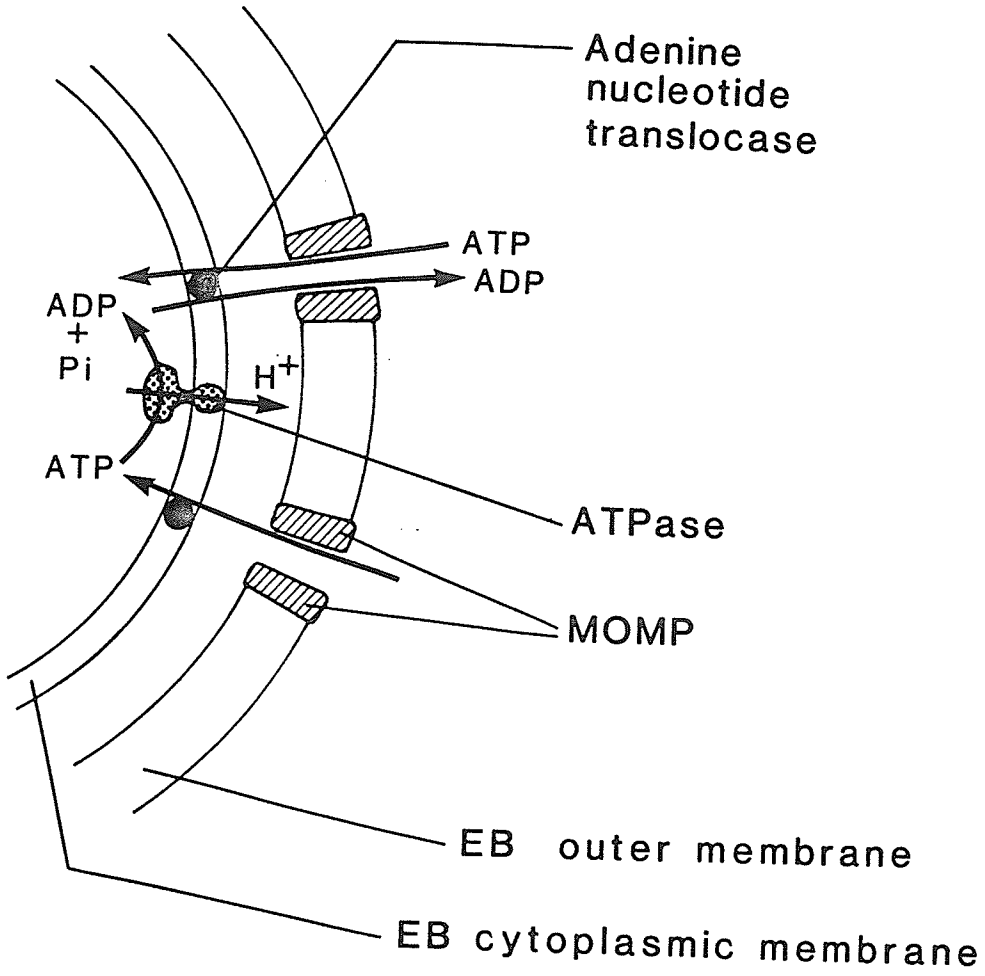


FIGURE 36. Hypothetical schematic model of MOMP porin activation in C. trachomatis EBs reduced with 2-ME. (Features were not drawn to scale)

these enzymes in a preformed state. The outer membrane of EBs in an oxidized environment is tightly cross-linked and impermeable. Once disulphide linkages are reduced, nucleotide triphosphate can freely transit the outer membrane. Thus under reducing conditions the presence of these precursor molecules may be sufficient to trigger the enzymatic activity. Hatch et al (1986) showed that MOMP of C. trachomatis was reduced to its monomeric form within 4 hours of infection in HeLa cells. Thus the reduction of MOMP has in vivo relevance in the EB to RB differentiation process. It may be that neutralizing anti-MOMP antibodies reduce chlamydial infectivity by limiting nucleotide permeation through MOMP pores.

The antibody mediated inhibition of porin function observed with chlamydiae may also have wider implications in anti-bacterial immunity. Porins are non-specific channel formers and a large number of them have been identified in gram-negative bacteria. Gabay and Schwartz (1982) used monoclonal antibodies directed against the Lam B protein as a probe for structure and function of the outer membrane of E. coli. They found two Mabs recognizing surface exposed epitopes that inhibited ^{14}C-maltose transport for E. coli. Though porins may not be as essential to other gram-negative bacteria as they are to C. trachomatis, they are still important in bacterial metabolism (Nikaido and Vaarta, 1985). The presence of antibody that blocks these porins could therefore be an important mechanism of

antibacterial immunity, especially in parts of the body where complement levels for bacteriolysis is low and/or phagocytes for opsonization are absent. It is hoped that this hypothetical model of energy exchange underlying EB to RB transformation will provide the basis for further experimental work into the role of the outer membrane proteins in the pathogenesis of Chlamydia trachomatis infection.

SUMMARY

The major conclusions from this study on the role of outer membrane protein in C. trachomatis infection of HeLa cells are summarized as follows:

1. C. trachomatis EBs made its initial contact with HeLa 229 cells predominantly through the microvilli.

2. C. trachomatis EBs did not appear to be internalized through a receptor-mediated process involving clathrin-coated pits.

3. Replicating RBs appear to marginate the inside of inclusion membranes around areas of dense mitochondrial and rough endoplasmic reticulum concentration. Its membrane appeared fused to the inclusion membrane in places.

4. Optimal conditions for in vitro neutralization assay were determined as: incubation at 37^{o}C for 30' in a small reaction mix ($\leq$ 400 ml) containing 10 ug/ml normal mouse IgG to prevent heat inactivation of EBs. A prozone effect was observed when antigen and antibody were not in equivalence.

5. Ninety-nine hybridomas were produced to antigens and screened for neutralizing activity. Three Mabs directed to serovar-specific and subspecies-specific epitopes on MOMP neutralized chlamydial infectivity for HeLa cells.

6. For monoclonal antibodies against MOMP, neutralization correlated with intensity of dot-blot reactivity and protection of mice from toxic death. Dot-blot reactivity reflects immunoaccessibility of the epitope on native EBs and high antibody avidity.

7. Neutralization of infectivity was not the result of inhibition of attachment or endocytosis but likely prevented EB intracellular development.

8. Trypsin treatment of EBs resulted in enhancement of infectivity for HeLa cells probably through increased outer membrane permeability. MOMP and 75 kDa were major targets of proteolysis.

9. 2-mercaptoethanol treatment of EBs also increased outer membrane permeability as indicated by RNA polymerase and ATPase activity.

10. Neutralizing MOMP Mabs inhibited RNA polymerase and ATPase activities suggesting that MOMP is the major porin protein in the outer membrane.

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