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Histopathology and ultrastructural pathology of chlamydiosis in turkeys and J774 cells

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Tappe, James P., Ph.D.
Iowa State University, 1991
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Histopathology and Ultrastructural Pathology of Chlamydiosis in Turkeys and J774 Cells

by

James P. Tappe

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY Major: Veterinary Pathology

Approved:

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For the Graduate College

Iowa State University
Ames, Iowa
1991
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GENERAL INTRODUCTION

Chlamydiae are gram negative intracellular bacteria that cause economically important diseases in humans, domestic mammals, and birds. There are 3 species; two, C. trachomatis and C. pneumoniae, primarily are human pathogens and the third, C. psittaci, is a pathogen of both humans and animals. Strains of C. trachomatis have, for several years, been known to cause distinct disease syndromes in humans and mice. Although these strains are antigenically related, serology, in combination with clinical signs, commonly is used for diagnosis of C. trachomatis infection. In contrast, infection by a specific strain of C. psittaci is difficult to diagnose because correlation of C. psittaci strains, serology and disease syndromes has not been accomplished.

Some strains of Chlamydia psittaci are associated with distinct disease syndromes in turkeys. The utility of these studies for establishing a diagnostically useful classification system is diminished by differences in chlamydial propagation techniques, dose of chlamydiae administered, route of inoculation, and times from infection to necropsy. Pathogenicity testing alone is expensive, inexact and technically difficult to reproduce but pathogenicity studies can, when done uniformly and in conjunction with classification techniques such as restriction endonuclease analysis, establish
the precision and efficacy of new serological tests.

Chlamydiae have a characteristic developmental cycle that
alternates between the infectious, metabolically-inactive
elementary body (EB) and the replicative, metabolically-active
reticulate body. Chlamydiae are unable to synthesize ATP; they
are considered "energy parasites". They reside and multiply
in a cytoplasmic endosome that does not fuse with lysosomes. Within these endosomes, protected from host defenses, they cause
persistent infections and produce and release antigens that
elicit immune responses that are deleterious to the host. In turkeys, chlamydial antigen is detectable in inflamed tissues
long after organisms are no longer recoverable (personal
observation).

Improper or inefficient processing of chlamydial antigens,
by antigen presenting cells, is suggested by the inconsistent
and highly variable immune response (in terms of time of onset,
duration, and intensity) seen in turkeys to chlamydial major
outer membrane. Antigen processing is the series of events by
which a cell modifies antigen into a form that is, when combined
with major histocompatibility complex molecules, recognizable by
T helper cells. Processing of antigens requires a series of
proteolytic steps dependent on precisely-ordered endocytic
events including endosomal acidification and endosomal-lysosomal
fusion. Inhibition of any of these events, as implied by
studies with *Listeria monocytogenes*, could block the formation
of an effective immune response.\textsuperscript{35}

The objectives of this study were: 1) to determine if one mammalian and two avian strains of \textit{C. psittaci}, each from distinct groups determined by restriction endonuclease analysis, cause specific pathologic syndromes in turkeys, and 2) to test the hypothesis that chlamydiae inhibit endosomal acidification and that this inhibition can be blocked by preexposing the chlamydiae to heat.

The National Animal Disease Center - National Veterinary Services Laboratories' Animal Care and Use Committee reviewed and approved the first study and determined that the animals were treated humanely in all procedures involved in that study.

The Ph.D candidate, James P. Tappe, was the principal investigator for each study.

**Explanation of Thesis/Dissertation Formation**

This dissertation is presented in the alternate format and consists of two manuscripts to be submitted to refereed scientific journals; the first has been submitted to Veterinary Pathology and the second will be submitted to Infection and Immunity. The format used is that of Veterinary Pathology. A literature review precedes the first manuscript and a general summary follows the second manuscript.
LITERATURE REVIEW

Chlamydiae

Taxonomy

Early schemes for classification of chlamydiae were complex and confusing because they were based on clinical or pathological syndromes, ability to grow in chicken embryos, or epidemiology. In 1968 Page proposed a classification system based on common antigenic, morphological, and developmental cycle characteristics of the organisms. He proposed that all members of this group be classified under the species Chlamydia psittaci and C. trachomatis. The current edition of Bergey's Manual of Systematic Bacteriology recognizes these two species. Recently, a third species of Chlamydia has been identified. Originally designated C. psittaci strain TWAR [after the laboratory designations of the first isolates - TW (Taiwan)-183 and AR (initials of a patient with pharyngitis)], this organism has been renamed C. pneumoniae based on ultrastructural morphology of the elementary body, DNA analysis, and serology. Chlamydia pneumoniae is one of the leading causes of pharyngitis and pneumonia in humans.

The microimmunofluorescence (MIF) test and restriction endonuclease analysis have been used to classify the numerous
strains of *C. psittaci*. Early MIF testing was done using sera from mice inoculated with individual chlamydial strains.\textsuperscript{52,53,114} Monoclonal antibodies have enhanced the specificity of the MIF test; mammalian strains are easily separated from avian strains.\textsuperscript{64,173} Immunoblotting analysis of chlamydial outer membrane polypeptide profiles (separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) also separates *C. psittaci* strains into avian and mammalian groups.\textsuperscript{63}

Within these two groups, distinct subgroups can be identified. Avian strains of *C. psittaci* can be subdivided into turkey, psittacine, pigeon, and duck serovars and mammalian (ovine) strains into abortion and polyarthritis serovars.\textsuperscript{7,9} MIF analyses were corroborated by restriction endonuclease analyses.\textsuperscript{7} Interestingly, although the serovar-specific monoclonal antibodies neutralized their homologous strain in an inclusion reduction assay, group-specific monoclonal antibodies (MAbs) did not.\textsuperscript{9} Additional mammalian serovars (feline pneumonitis, guinea pig inclusion, muskrat, and cattle) have recently been identified by serovar-specific MAbs in the MIF test.\textsuperscript{8} Analyses frequently result in placement of strains into groups corresponding to the species from which they originally were isolated (i.e. biotypes).\textsuperscript{8,114} This suggests that elementary body outer membrane proteins are important in determining host (and often disease syndrome) specificity.
Structure and metabolism

Chlamydial elementary bodies are enveloped by an outer cell wall and a plasma membrane. The surface of the cell wall is focally interrupted by a patch of 10-30 rosette-like pores through which protrude surface projections. These projections extend to the chlamydial cytoplasmic membrane and may be connected to intracytoplasmic DNA strands. The function of these projections is unknown but they may be involved in attachment of the elementary body (EB) to the host cell.115

The major components of the cell wall are the genus-specific lipopolysaccharide (LPS) and the major outer membrane protein (MOMP). The MOMP contains cysteine residues that allow the formation of disulfide bonds, giving rigidity to the cell wall. These disulfide bonds also block the pore function/activity of the cell wall resulting in resistance to changes in osmotic pressure. Exposure of the EB to reducing conditions (i.e. as would be present in early endosomes) breaks the disulfide bonds, makes the cell wall less rigid, and opens pores--features necessary for intracellular growth and replication. It was calculated that pore size after reduction of disulfide bonds was large enough for passage of nucleotide triphosphates; chlamydiae cannot synthesize their own adenosine triphosphate (ATP), and EBs (vs. reticulate bodies) cannot take up ATP.18,81,187

Sequence analysis of C. psittaci MOMPs has revealed 4
variable domains. Variable domains I, II, and IV contain serovar specific and subgroup specific epitopes. The variable domains are the only part of the MOMP exposed on EBs. Three of the 7 cysteine residues in the \textit{C. psittaci} MOMP are between variable domains.

Chlamydial lipopolysaccharide (LPS) resembles the LPS of other gram-negative bacteria; it is composed of lipid A and 2-keto-3-deoxyoctonic acid (KDO) and has endotoxin-like activity. The LPS contains at least 2 epitopes; one cross-reacts with \textit{Salmonella} rough mutants and \textit{Acinetobacter calcoaceticus}, and the other epitope is specific to the genus \textit{Chlamydia}. Injection of EBs intravenously into endotoxin-resistant (C3H/HeJ) mice resulted in a condition mimicking endotoxic shock. This finding, plus the ability of mild heat treatment to abolish EB-induced cytotoxicity (in tissue culture), suggests that the toxicity of chlamydial EBs is not due to LPS.

In contrast to other gram negative bacteria, chlamydiae lack peptidoglycan but contain penicillin-binding proteins in their cell wall. The presence of penicillin-binding proteins, and the inhibitory effect of penicillin on chlamydiae, suggest that there is cross-linkage between peptides and a nonpeptidoglycan-containing polysaccharide in the chlamydial cell wall.

Chlamydiae lack the biochemical pathways for production
of ATP.\textsuperscript{187,188} Elementary bodies, when treated with reducing agents, and reticulate bodies acquire ATP from the host cell by an ATPase that exchanges ADP for ATP.\textsuperscript{81,145} Reduction of MOMP cysteines on EBs (or cysteines on a 60kd protein associated with MOMP) may result in the opening of a pore allowing entry of ATP; MAbs against MOMP block ATPase activity.\textsuperscript{145} Hydrolysis of ATP may result in an electrochemical gradient across the EB cytoplasmic membrane; this gradient could be used to drive nutrient (i.e. amino acids) transport into the reticulate body.\textsuperscript{81} The chlamydial ATPase is inhibited by oligomycin (an F\textsubscript{1}F\textsubscript{0} ATPase inhibitor) but not ouabain (a Na\textsuperscript{+},K ATPase inhibitor).\textsuperscript{81,144}

Amino acid requirements of chlamydiae depend on the chlamydial strain and the concentrations of antagonistic amino acids in the environment.\textsuperscript{37} The meningopneumonitis strain of \textit{C. psittaci}, in the presence of ATP, incorporated significant amounts of carbon into its molecules from only 2 (asparate and isoleucine) of 15 amino acids tested.\textsuperscript{188} Interferon-induced inhibition of chlamydial replication probably results from decreased levels of tryptophan (due to induction of indoleamine-2,3-deoxygenase), resulting in a \textit{relative} increase in the antagonist phenylalanine.\textsuperscript{26,37}
Developmental cycle

An active area of chlamydial research is the mechanism of uptake of EBS by the host cell. The phrases "parasite-specified phagocytosis" and "smooth membrane pathway" have been used to describe endocytosis of chlamydiae. Endocytosis is a general term for the uptake, by cells, of fluid, and of subparticulate and particulate material. Proposed subtypes of endocytosis are "phagocytosis", "pinocytosis", and "receptor-mediated endocytosis".

Several findings support the argument that these terms and phrases really represent a single, clathrin-dependent process (or at most 2 processes) with features of all of the proposed endocytic subtypes. For example, the formation of clathrin-coated vesicles is independent of particle size or the presence of specific receptors. The findings 1) that soluble, fluid-phase markers and receptor-bound ligands are cointernalized into the same endosome, and 2) that endosomes formed by either "receptor-mediated" or "fluid phase" endocytosis acidify their lumens, are further evidence for a single endocytic process.

An alternative process, uptake into non-clathrin-coated endosomes, has been proposed but not thoroughly studied. The formation of both coated and noncoated endosomes is reduced when cells are incubated with cytochalasin B. Endocytosis of material into noncoated endosomes may simply represent an
overflow or "default" pathway where ligand-bound receptors are not concentrated (vs. the clathrin-induced clustering of receptors) before internalization. Irrespective of the type of endosome into which ligands originally enter, all can follow a common intracellular pathway.

The studies cited above allow one to conclude that the internalization mechanism, per se, does not determine the intracellular fate of chlamydiae. Unfortunately, the artificial subdivision of the endocytic process, and the overinterpretation of morphological observations, has led to conclusions supporting the importance of the internalization process in the intracellular survival of EBs. I believe that differences in intracellular destinations of EB-containing endosomes simply depends on the inclusion (or exclusion) in the endosomal membrane of specific plasma membrane proteins in relative or absolute quantities.

The intracellular fate of an elementary body is highly dependent on its mode of attachment to the host cell membrane. Proposed receptors for nonopsonized EBs include: 1) macrophage complement receptors (C3R, C4R), 2) the lectin-like mannosyl/fucosyl receptor on macrophages, and 3) receptor(s) for the EB (vs. reticulate body)-specific, 18kd and 31kd (16kd and 29kd on C. psittaci), trypsin-resistant, heparin-binding proteins of C. trachomatis. The receptor(s) for these latter proteins is found on a variety of nonphagocytic
cells and internalization via attachment to this receptor(s) apparently results in a productive infection.190

Because trypsinization of EBs did not inhibit infectivity for Hela cells, it was concluded that the MOMP was not involved in attachment of the EB to the host cell.77 However a later study with a more protease-sensitive strain suggested the MOMP was involved in attachment of EBs to cells.172 This study was corroborated by the finding that the neutralization of immunoaccessible MOMP epitopes, by monoclonal antibodies, blocked infectivity of EBs for monkey conjunctivae.199 The adhesive properties of the MOMP reside in variable domains II and IV; these properties are nonspecific and probably involve both electrostatic and hydrophobic interactions with host cells.171

Although chlamydial LPS is surface-exposed, evidence suggests that it is not involved in attachment of EBs to host cells.199 The finding that an LPS epitope present on reticulate bodies was undetectable on EBs suggests that chlamydial lipopolysaccharide is partially buried in the EB outer membrane.36 It is possible that the LPS has a minor role in attachment by promoting hydrophobic interactions between the EB and the host cell.183

Opsonic phagocytosis of EBs can be mediated by macrophage Fc or complement receptors. Rate of uptake of chlamydiae was similar for EBs incubated in either immune or nonimmune serum.
suggesting that EBs may attach to Fc receptors via adsorbed, nonspecific antibodies. Uptake of chlamydiae was inhibited by the preincubation of macrophages with antibodies against complement receptors, but the presence of complement did not enhance attachment of EBs, indicating that chlamydiae may directly bind to complement receptors.\textsuperscript{155}

Based on comparisons with \textit{E. coli} and polystyrene beads, the phrase "parasite-specified phagocytosis" was coined to describe the endocytosis of chlamydial elementary bodies by so-called "nonprofessional phagocytes" (i.e. L and Hela cells).\textsuperscript{27} This phrase was chosen to indicate that the phagocytosis of EBs was somehow facilitated by the chlamydiae themselves; they were taken in nearly 100 times faster than the beads or than \textit{E. coli}. The authors also indicated that this process involved the attachment to ubiquitous structures on the host cell surface. It has been suggested that the increased rate of chlamydial ingestion may simply reflect higher numbers of adhesins on the EB (vs. polystyrene beads or \textit{E. coli}) rather than an active metabolic role by the EB. Increased numbers of adhesins would promote more efficient formation of endosomes by the host cells.\textsuperscript{184}

Most evidence favors uptake of chlamydiae by a clathrin-independent process. The involvement of clathrin-coated pits in the uptake of chlamydiae has been inferred by morphological studies, by studies in which cytosolic acidification was used
to prevent the formation of coated pits, and by studies using calmodulin inhibitors.\textsuperscript{86,153} Calmodulin inhibitors reduce the uptake of EBs; calcium-activated calmodulin is necessary for the recruitment of clathrin to the plasma membrane.\textsuperscript{128,159,160} Endocytosis of \textit{C. psittaci} EBs usually is associated with the formation of coated pits but \textit{C. trachomatis} EBs may also use a so-called "smooth membrane pathway".\textsuperscript{153} No attempt was made to confirm the presence or absence of clathrin (i.e. by immunogold technique with anti-clathrin antibodies). In a later study that incorporated immunoelectron microscopy, clathrin frequently was found associated with pits and endosomes containing EBs.\textsuperscript{86} The inability to detect clathrin-coated pits may reflect the culture conditions of the host cells, the method of processing tissue for electron microscopy or the plane of section.\textsuperscript{86,195} Clathrin was found associated with at least part of every EB-containing endosome when serial sectioning was done.\textsuperscript{86} I believe that these findings suggest that only focal patches of clathrin are necessary for endocytosis of larger particles such as EBs.

Although earlier work suggested that ingestion of chlamydiae was independent of microfilaments, this was later refuted when it was found that cytochalasin D inhibited chlamydial endocytosis by Hela cells.\textsuperscript{72,184} Coated vesicle formation is inhibited by cytochalasins.\textsuperscript{160} The presence of a microfilament-independent mechanism of uptake cannot be
completely ruled out because in some experiments EBs entered cells in the presence of cytochalasin D.\textsuperscript{148,153}

The most important, and probably interrelated, subsequent steps in the chlamydial developmental cycle are the formation/expansion of the inclusion membrane, the acquisition of ATP by the EB, and the inhibition of phagolysosomal fusion. Obviously the initial inclusion membrane is derived from the host plasma membrane. Differences in the protein content of membranes from phagosomes containing either live or heat-inactivated EBs support the hypothesis that the intracellular fate of an EB is determined, at the time of its attachment to the host cell, by specific proteins incorporated into (or excluded from) the phagosomal membrane. Phagosomal membranes surrounding live EBs contained a 70 kilodalton (kd) protein not found in membranes of phagosomes containing heat-inactivated EBs. Conversely, phagosomal membranes surrounding live EBs were devoid of a 25 kd protein found in membranes of phagosomes surrounding heat-inactivated EBs and had reduced amounts of 36 kd and 38 kd proteins.\textsuperscript{197} Because of the methods used it was difficult to determine whether the phagosomal membrane proteins were derived from the host plasma membrane or from the chlamydiae; at least some phagosomal membrane proteins shared electrophoretic mobilities with plasma membrane proteins. In addition, most of the proteins from membranes surrounding live EBs had the same electrophoretic
mobilities as those from membranes of phagosomes containing metabolically-inactive (heat-inactivated) EBs. Of interest was the finding of very similar protein electrophoretic patterns from EB phagosomal membranes from different cell types even though the plasma membrane protein patterns were dissimilar. This is compatible with the use, by various cell types, of a common mechanism of EB phagocytosis. Cells infected in the presence of chloramphenicol (which inhibits chlamydial but not host cell protein synthesis) have EB-containing endosomes that do not fuse with lysosomes, a finding that supports the hypothesis that plasma membrane-derived, not chlamydia-derived, proteins determine the fate of the EB endosome in the host.

The common usage of cycloheximide in cell cultures supporting chlamydial growth suggests that the enlargement of the chlamydial inclusion membrane is not due to active addition of protein components by the host cell. Instead, additional membrane must be acquired either from components synthesized by reticulate bodies or by fusion with preexisting host cell golgi or endoplasmic reticulum-derived membranes. The frequent finding of chlamydial inclusions in close proximity to, or in contact with, the endoplasmic reticulum and mitochondria suggests that these organelles may contribute to expansion of chlamydial inclusion membranes. On the other hand, membrane derived from vesicles commonly seen
within chlamydial inclusions may contribute to enlargement of the inclusion membrane. These vesicles are enriched in LPS and may be the source of chlamydial LPS found in the plasma membrane of infected and adjacent cells in culture. Toxoplasma secrete, from their rhoptries, vesicles that modify the membrane of their phagosomes; this modification may prevent fusion with lysosomes.

The uptake of ATP by EBs is dependent on reduction of disulfide bonds in the EB outer membrane, resulting in formation of pores. Reduction of disulfide bonds, possibly mediated by glutathione:protein-disulfide oxidoreductase, can occur at the surface or in early endosomes of the host cell. Pores allow access of ATP molecules to the EB's plasma membrane where, hypothetically, they contact nucleotide translocases. These translocases, possibly in conjunction with an electrochemical gradient established by an ATPase, then facilitate diffusion into the chlamydial cytoplasm which promotes conversion of the EB into a reticulate body.

The acquisition of ATP by reticulate bodies requires that adenosine nucleotides cross at least 3 barriers: the inclusion membrane and the outer and plasma membranes of the bacterium. The permeability of the inclusion membrane to nucleotides is unknown. It is possible that ATP molecules cross by simple diffusion. The frequent observation of attachment of mitochondria to chlamydial inclusion membranes suggests, to
me, that there is facilitated or active transfer of ATP across the membrane. This may be accomplished by a modification of the reticulate body membrane that mediates its attachment to the inclusion membrane. The electron microscopic observation of protrusion of mitochondrial membranes through the inclusion membrane suggests chlamydiae also may recruit enzyme systems for production of ATP within the inclusion (personal observation).

Whether or not chlamydiae inhibit phagolysosomal fusion depends on: a) an unaltered EB outer membrane and b) the type of cell infected, but not the exposure of the host cell to cytokines. The ability of isolated EB cell envelopes to temporarily inhibit phagolysosomal fusion implies that the early stages of this process are determined during the initial stages of EB endocytosis. This initial inhibition of phagolysosomal fusion has been hypothesized to be the result of retention of clathrin around the EB-containing endosome. The finding that antibody or heat treatment of EBs results in fusion of their endosomes with lysosomes supports the hypothesis that initial interactions of the EB outer membrane with the host plasma membrane determine the fate of the endosome.

Chlamydiae do not inhibit phagolysosomal fusion in human polymorphonuclear cells in vitro. This lack of inhibition is independent of opsonization or heat inactivation; the
mechanism is unknown. So far, no other cell type has been shown to possess this property.

Fusion of EB-containing endosomes with lysosomes apparently is not promoted by gamma interferon or tumor necrosis factor. However, exposure of infected cells to these cytokines results in inhibition of chlamydial growth and replication by inhibiting the transformation of EBs into reticulate bodies\(^{44,45,112}\). The mechanism of this inhibition is the interferon-induced depletion of host cell tryptophan—probably via induction of indoleamine-2,3-dioxygenase.\(^{26}\) Tumor necrosis factor causes its effect by inducing the production of interferon.\(^{164}\)

The mechanism of release of chlamydiae from cells involves the release of host cell lysosomal enzymes.\(^{176}\) These enzymes would cause the breakdown of the inclusion membrane which precedes cell lysis.\(^{31}\) Some strains of C. trachomatis are released by exocytosis without concomitant death of the host cell.\(^{175}\)

Antigen Processing

An effective immune response requires presentation of antigen to T cells in association with major histocompatibility complex (MHC) molecules. In most cases "native" antigen must be processed to a small peptide before
it can be presented. Antigen processing is complex and involves endocytosis, exocytosis and several intracellular compartments.

The initial step in processing of most antigens is uptake into an endosome. The route taken by the antigen after uptake is antigen/organism-specified but the physicochemical characteristics responsible for this specificity are unknown. Generation of antigen fragments is time dependent and, in the case of B cells, the types of fragments produced are dependent on the specificity of the mIgG expressed, or whether or not antigen is bound by antibody.

Acidification of endosomes is critical for processing of some types of antigen. The presentation of antigen to T cells can be inhibited by neutralization of acidic endosomes by chloroquine or ammonium chloride. Endosomal acidification is mediated by proton pumps which are ATP dependent. This process occurs in 2 steps and results in populations of "early" and "late" endosomes with average pHs of 6.2 and 5.3, respectively. Processing occurs in both of these compartments as well as in lysosomes. Defects in proton pump assembly lead to inappropriate sorting of lysosomal enzymes and inhibition of lytic processing of proteins. Dissipation of lysosomal acidity decreases transport of cysteine into the lysosome. Cysteine facilitates lysosomal antigen processing by reduction of protein disulfides.
At least 3 pathways of antigen processing, one resulting in presentation with MHC class I (MHC-I) molecules and 2 resulting in presentation with MHC class II molecules (MHC-II), have been proposed or described.

**Presentation with MHC-I**

Processing for MHC-I presentation begins with escape of antigen from the endosome into the cytosol (or direct entrance into the cytosol as with some viral, i.e. paramyxovirus fusion protein, antigens) where it may bind with heat shock protein (HSP). Some studies indicate that heat shock proteins are necessary for transport of antigen into the endoplasmic reticulum. Evidence for HSP-independent peptide transport across the membrane of the rough endoplasmic reticulum (RER) is supported by the finding of ATP-dependent transmembrane transport protein genes in the class II region of the MHC gene complex. Mutations or deletions in these genes results in inefficient assembly of class I proteins in the RER (a process dependent on presence of peptide) and decreased longevity of the MHC-I molecule on the surface of the cell (addition of exogenous peptide stabilizes the molecule). Alleles at the region of the transporter protein gene locus change the specificity of class I antigen presentation suggesting that these proteins influence the cell mediated...
immune response. Antigen has 2 fates in the RER: combination with MHC-I (in conjunction with HSP), or degradation.6,40,134 Excess T-cell receptor subunits are degraded in the RER.105 Generation of immunogenic peptides from native antigen could occur in the cytoplasm or RER. Displacement of a chaperonin protein (probably from the a1 and a2 domains of the heavy chain) and binding of peptide with MHC-I heavy chain induces association with B2 microglobulin and release from the RER.3,88,177 From here they are transported, probably via golgi apparatus, to the cell surface.177 The rate-limiting step in presentation of antigen with MHC-I probably is transport of peptides into the RER.

Presentation with MHC-II

Antigen presentation with MHC-II molecules begins with endocytosis of antigen into a vesicle that is progressively acidified. Processing can occur in both endosomes and lysosomes; processing in lysosomes results in more efficient antigen presentation. Bacteria probably require lysosomal processing.79 Cells defective in endosomal acidification have decreased levels of antigen presentation to CD4+ T cells.118 Formation of MHC-II-antigen complexes is accelerated at pH levels (pH 5) similar to those seen in antigen presenting cell endosomes.93 Acidification activates enzymes, including
cathepsins B and D, that are important in antigen processing and presentation. Cathepsin B may be involved in antigen proteolysis and cathespin D (along with a leupeptin-sensitive enzyme) may function to remove the invariant chain protein from MHC-II molecules, allowing them to complex with antigen.\textsuperscript{22,132} Chloroquine inhibits invariant chain dissociation from MHC-II molecules.\textsuperscript{132}

Cells require invariant chain protein for the presentation of some antigens with MHC-II molecules.\textsuperscript{78,170} This protein also is necessary for targeting of MHC-II molecules to endosomes.\textsuperscript{75,107,109} These RER-derived endosomes transfer MHC-II molecules to the golgi apparatus for further modification. Golgi-derived endosomes, containing MHC-II molecules, fuse with endosomes containing antigen and cathepsins.\textsuperscript{129} Progressive acidification results in activation of cathepsin D, in turn resulting in removal of invariant chain from MHC-II molecules.\textsuperscript{118} This may allow a conformational change or may simply uncover the antigen binding site allowing antigen to associate with the MHC-II molecule.

Recycling of surface MHC-II molecules also may be an important source of receptors for processed peptides; there may be exchange of peptides (i.e. old for new) in MHC binding sites, but the role of recycled MHC-II molecules in antigen presentation is unclear.\textsuperscript{1,80,110} Recently it has been shown that both MHC-I and -II molecules recycle through a pathway that is
sensitive to primaquine. Recycling of MHC molecules may be nothing more than a laboratory curiosity; methods that do not cause extensive, nonspecific crosslinking of surface proteins (thus inducing nonspecific endocytosis) are unable to detect MHC recycling.42

Another pathway of antigen presentation with MHC-II molecules, proposed on the basis of its resistance to chloroquine and sensitivity to brefeldin A, is the so-called "endogenous pathway". In this pathway, which requires high doses of antigen and long infection times, antigen introduced into the cytoplasm is transported with heat shock protein to the RER where it combines with the MHC-II molecule.91 It has been suggested that invariant chain protein does not block binding of antigen to MHC-II in the RER.133 Another possibility is that some of the tested cell lines express little or no invariant chain protein.90

Endosomal acidification

Acidification of endosomes is mediated by H+-ATPases. These proton pumps are from 1 of 3 families of ATPases that are important in maintaining ion gradients or generating ATP. F0F1H+-ATPases include the mitochondrial enzyme that generates ATP. P-ATPases function via an intermediate that has its active site phosphorylated. This family includes the Na+-K+-ATPase of the plasma membrane, the Ca2+-ATPases of the
sarcoplasmic reticulum and plasma membrane, and the H+\textsuperscript{+}-K+\textsuperscript{+}-ATPase of the gastric mucosa. Endosomal ATPases are in the V-ATPase (vacuolar ATPase) family and are found on endosomes, lysosomes and the golgi apparatus. They function to generate proton and electrical gradients across the membranes of these organelles.\textsuperscript{58,130}

The V-ATPase is composed of 9 subunit types with molecular weights of 17,000 - 100,000. Each ATPase complex contains 3 copies of a 73,000 m.w.(73K) subunit located on the cytoplasmic surface of the vesicle where they bind and hydrolyze ATP. Six copies of a 17,000 (17K) m.w. subunit form a transmembrane proton channel.\textsuperscript{11,12,14} Two other ATP binding sites, possibly important in regulation, are present on the molecule but their subunits have not been identified. There are also binding sites for SO\textsubscript{4}\textsuperscript{2-} and NO\textsubscript{3}\textsuperscript{-} which, when occupied, inhibit the ATPase activity of the molecule. Binding sites for I\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-}(in the presence of ATP), when occupied, cause dissociation of the molecule. N-ethylmaleimide (NEM) and SCN\textsuperscript{-} ions, by an unknown mechanism, also directly inhibit the proton-pumping capacity of the molecule.\textsuperscript{15,60}

The pH differences of endosomal subpopulations, determined by differences in permeability to cations and anions, likely control membrane trafficking and the efficiency and pathways of receptor and ligand routing in the cell.\textsuperscript{60} Conditions that increase efflux of internal cations facilitate
endosomal acidification. Early, but not late, endosomes contain a Na+,K+-ATPase that limit early endosomal acidification by creating an interior positive charge that opposes H+ influx. This charge is created by pumping in 3 Na+ ions in exchange for 2 K+ ions. Ouabain, which binds to the endosomal lumen-oriented alpha subunit of the Na+,K+-ATPase, promotes early endosomal acidification.\textsuperscript{60,61}

Constant proton pumping (required because of the high permeability of the endosomal membrane to protons) results in an electrochemical gradient that limits endosomal acidification. Inward movement of chloride ions, through a channel which is not a component of the proton pump, neutralizes the gradient and promotes acidification.\textsuperscript{13,60,181} That this channel is important in regulating endosomal pH is demonstrated by the inhibition of endosomal acidification by blocking Cl\textsuperscript{-} conductance. Chloride influx is controlled by phosphorylation of the channel which is mediated by a cAMP-dependent protein kinase A. Dephosphorylation, by alkaline phosphatase, inhibits chloride influx and endosomal acidification. The chloride channel and the proton pump are colocalized on the endosomal membrane.\textsuperscript{15}

**Endosomal fusion and trafficking**

The mechanisms of early and late endosomal fusion differ (Appendix, Table 1). Results from cell-free assays show that
early endosomes fuse with each other but not with late endosomes. These findings were supported by studies with intact cells and extended to show that the ability of early endosomes to fuse with one another decreases with time (t½=8 min.). Early endosomal fusion is dependent on KCl in the medium (apparently a K⁺-specific mechanism); KCl is not required for fusion of late endosomes with lysosomes. N-ethylmaleimide (NEM)-sensitive cytosolic proteins are necessary for fusion of early endosomes with one another but not for fusion of late endosomes with lysosomes. Early endosomal interactions, in contrast to those between late endosomes and lysosomes, are independent of microtubules. The requirement of intact microtubules has hampered the development of satisfactory models for in vitro study of late endosomal interactions with lysosomes. Early and late endosomes have different membrane cholesterol concentrations and unique and shared membrane proteins.

The hypothesis that endosomal acidification is necessary for fusion of endosomes is supported by the finding that transfer of various ligands from endosomes to lysosomes is inhibited by neutralizing endosomal pH. This hypothesis also is supported by the finding that several microorganisms (i.e. Toxoplasma gondii, Glugea Hertwigi, Legionella pneumophila, Mycobacterium tuberculosis) that cause inhibition of fusion of their phagosomes with lysosomes also inhibit
endosomal acidification. Neutralization of *G. hertwigi, T. gondii,* and *L. pneumophila* with, respectively, cationized ferritin, antibodies or heat, or formalin allows acidification of their endosomes and phagolysosome fusion. The ability of *Rickettsia mooseri, Toxoplasma gondii,* and chlamydiae to survive in endosomes can, by an unknown mechanism, be affected by preincubation of the organisms with immune serum or monoclonal antibodies. Ultrastuctural analyses show that endosomes containing either antibody-coated toxoplasma or chlamydiae fused with lysosomes. Fusion of digestive vacuoles (DV) with lysosomes occurs only after DV acidification in *Paramecium caudatum.* Acidification may cause modification of the DV membrane permitting fusion with lysosomes.

It has been suggested that antibody-coated *T. gondii* inhibit phagolysosomal acidification in cells with Fc receptors by exclusion of the proton pump from the endosome membrane upon endocytosis. It was also suggested that phagolysosomal fusion in this system was independent of endosomal acidification, but this was not conclusively demonstrated. *Toxoplasma* secrete rhopterie or surface-derived vesicles, within 5 minutes after phagocytosis, that form an intraphagosomal network connecting the surface of the organism with the phagosomal membrane. This network is produced by live, but not heat-killed or antibody-coated,
toxoplasmae. Modification of the phagosomal membrane by this network may play a role in the ability of toxoplasmae to inhibit endosomal acidification.\textsuperscript{165} Phagosomal membrane modification also has been suggested as a mechanism by which algae (Chlorella) inhibit P-L fusion in paramecia (Paramecium bursaria).\textsuperscript{98}

Dissipation of endosomal pH does not inhibit fusion of endosomes in some assays.\textsuperscript{24,50} In one study, concentrations of NH$_4$Cl and chloroquine in the culture medium were 2.5 and 6 times lower than typically used to neutralize acidic endosomes.\textsuperscript{10,152} The response to neutralizing agents such as ammonia and chloroquine is dependent on their concentration; fusion is inhibited at some concentrations and stimulated at others.\textsuperscript{46} I believe that fusion of the mildly acidic early endosomes is independent of, or just facilitated by, low pH. Acid pH may be more important in the sorting away of membrane proteins (that inhibit late endosomal acidification or fusion with lysosomes) at the compartment of uncoupling of receptor and ligand (CURL) and/or in fusion of late endosomes with lysosomes. Trafficking of endosomes to their appropriate destinations would be facilitated by different fusion requirements.

The inhibition of fusion of Fc-receptor-containing, J774 cell-derived, early endosomes with other endosomes, by NEM and by mild trypsinization, demonstrates that proteins in the
cytosol, and on the cytosolic surface of the endosomes, are necessary for the fusion process. Recently, several genes have been discovered that are involved in endosomal trafficking and fusion in the secretory pathway of yeasts. These secretory pathway (SEC) genes code for the cytoplasmic proteins NSF (N-ethylmaleimide-sensitive fusion protein) and SNAP (soluble NSF attachment protein) and the endosome-bound rab (ras genes from rat brain) proteins. The SEC4 subfamily codes for the GTP-binding rab proteins, 30 of which may exist in MDCK cells. Three of the rab proteins have localized to distinct endosomal compartments: rab 2 to the endoplasmic reticulum salvage compartment, rab 5 to early endosomes and rab 7 to late endosomes. So far, rab proteins have not been identified on lysosomes. The presence of endosomal-specific rab proteins suggests that these proteins play a role in the directional movement of endosomes in the cell (i.e. early endosome-CURL-late endosome-lysosome). The inhibition of endosomal fusion by GTP-gamma-S, in cell-free assays, suggests that GTP-binding proteins also are involved in the fusion process. Whether or not these latter proteins are rab proteins is unknown.

NSF protein probably is directly involved in the fusion of endosomes. There is increased fusion of vesicles when NSF is added to endocytic vesicle preparations derived from J774 and CHO cells. Addition of anti-NSF antibody or NEM inhibits
fusion of endosomes in this assay. The mechanism of NSF action is unknown but sequence analysis failed to detect hydrophobic regions suggesting that the protein does not "embed" in the lipid membrane of the endosome as do some viral fusogenic proteins. Attachment of the NSF protein to the endosomal membrane probably is mediated by SNAPs; this protein is required for attachment of NSF to golgi vesicle membranes and for fusion of these vesicles to membranes surrounding golgi cisternae.

A role for protein kinases in the fusion of endosomes has been proposed. A cytoplasmic protein (cdc2 protein kinase), present in Xenopus eggs arrested in mitosis, inhibits fusion of BHK cell endosomes in a cell free assay.

Turkey Ornithosis

Avian respiratory anatomy and physiology

The avian nasal cavity is roughly divided into compartments according to the concha the compartment contains. These compartments are the vestibular (contains the rostral concha), respiratory (contains the middle concha) and olfactory (contains the caudal concha). The infraorbital sinus, situated lateral to the nasal cavity, is enclosed almost totally by soft tissue walls except dorsocaudally where the caudal nasal concha forms part of the wall.
The lateral nasal glands provide moisture for the mucosa of the nasal cavity. They lie lateral to each nasal cavity deep in the submucosal connective tissue; their ducts empty into the vestibular compartments. They secrete salt in marine birds but in most other avian species they have lost this function.

The trachea divides at the syrinx into the extrapulmonary primary bronchi. These enter the medial surface of the lungs and continue to the caudal limits as the intrapulmonary primary bronchi (IPB). Four groups of secondary bronchi originate from the IPB. The medioventral and mediodorsal groups give off parabronchi that interconnect resulting in a system that supplies gas to the cranial and dorsomedial portions of the lung. The laterodorsal and lateroventral groups give off parabronchi that supply the rest of the lung but all of the secondary bronchial groups give off some parabronchi which interconnect with all of the other groups. The secondary bronchi (except the medioventral group) and parabronchi are lined by simple squamous epithelium.

The parabronchus forms the core of the avian pulmonary lobule. Numerous atria originate from the parabronchus and these, in turn, give off infundibulae that terminate in air capillaries. All of these structures are lined by a simple squamous epithelium which is covered with surfactant-like material, composed predominantly of dipalmitoyl
phosphatidylcholine, which originates from the osmiophilic bodies of granular cells of the parabronchi and atria.\textsuperscript{38,62,108,168} The air capillaries alternate with blood capillaries; blood and air flow in opposite directions forming a countercurrent system resulting in very efficient gas exchange.\textsuperscript{111}

Turkeys have 7 air sacs: the cervicoclavicular sac, paired medial clavicular, cranial thoracic, and abdominal sacs. A finding unique to turkeys is the absence of caudal thoracic air sacs.\textsuperscript{101} Air sacs are connected to the lungs via ostia that contain clusters of orifices of secondary bronchi and saccobronchi. Saccobronchi are formed by the coalescence of several parabronchi. The end of the intrapulmonary primary bronchus opens into the abdominal air sac.\textsuperscript{39,102} The air sacs are lined by a simple squamous epithelium.

Inhaled air takes two routes from the IPB; part of the air goes to the portion of the lung supplied by the mediodorsal group of secondary bronchi and part goes to the abdominal air sacs via the IPB. During expiration the gas in the abdominal air sacs perfuses the lungs – also via the mediodorsal secondary bronchi.\textsuperscript{102} This suggests that inhaled particles would first deposit in the dorsomedial and cranial portions of the lungs and in the abdominal air sacs. However, a study of inhaled radioactive particles in chickens found no difference between the amount of radioactivity in dorsal and ventral portions of the lungs, and a higher level of
radioactivity in the caudal portion of the lungs. Retention of radioactive particles for up to 36 hours probably was due to the lack of a mucociliary apparatus in the deeper regions of the lungs and air sacs and due to the low numbers of macrophages in the turkey respiratory tract.⁵⁶,¹²⁰

Pathogenicity of chlamydial strains for turkeys

In the 1950's and 1960's, before the advent of molecular biological techniques allowing precise classification of chlamydial strains, several pathogenicity studies were done to define the role of different chlamydial serovars in turkey ornithosis. Strains used were chosen either because they were isolated from turkeys in chlamydial outbreaks or on the basis of their virulence for laboratory animals. These studies used varying chlamydial propagation techniques, host environmental conditions, routes of inoculation, and numbers of chlamydiae inoculated thus diminishing comparability of findings.

These investigations were concerned only with gross and/or light microscopic lesions and sometimes cultural findings. Some authors only reported findings caused by one chlamydial strain. Davis and Delaplane, and Beasley described lesions caused by a virulent "Jo" (Texas turkey) strain of Chlamydia psittaci inoculated intratracheally.¹⁹,⁴³,¹⁴² The lesions were in turkeys of various ages which were infected
only one or two weeks. The following lesions were seen.

<table>
<thead>
<tr>
<th>Gross</th>
<th>Histologic</th>
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<tbody>
<tr>
<td>pericarditis</td>
<td>pneumonitis</td>
</tr>
<tr>
<td>pneumonia</td>
<td>bronchopneumonia</td>
</tr>
<tr>
<td>air sacculitis</td>
<td>tracheitis</td>
</tr>
<tr>
<td>peritonitis</td>
<td>arteritis</td>
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<tr>
<td>perihepatitis</td>
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<td>splenomegaly</td>
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<td>orchitis</td>
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Gale also used turkeys of various ages and described lesions up to 30 days post infection. He used a chlamydial strain of low virulence (inoculated by several routes) but found gross lesions similar to those in Davis and Delaplane's report. One difference was the absence of gross and histologic changes in the spleen and the absence of hepatitis or nephritis. No mention was made of lesions involving arteries or reproductive organs.

Another report by Beasley compared the histopathology in 3, 10, and 18-week old turkeys induced by 9 strains of chlamydiae. The turkeys were inoculated intratracheally with organisms contaminated by amnioallantoic fluid and necropsied 2 weeks later. Virulent chlamydial strains (originally isolated from turkeys) caused lesions similar to those described in Beasley's earlier report. Less virulent strains
(including strains originally isolated from psittacine birds) consistently produced lesions in the respiratory system and serous membranes (like virulent strains) but only infrequently in the heart, liver and spleen.

Page inoculated turkeys by various routes and compared the pathogenicity of several chlamydial strains.\textsuperscript{137,138,139} His findings were limited to gross lesions and cultural studies. Turkeys inoculated with a virulent strain (NJ-1) frequently died and had lesions similar to those reported by Davis and Delaplane.\textsuperscript{43} Other strains tested [pigeon ornithosis, bovine abortion (WC,EBA), lamb abortion, lamb arthritis, bovine encephalomyelitis (McNutt)] caused several types of lesions but did not cause death. The lesions induced by the bovine abortion (WC) and pigeon strains resembled those produced by the virulent turkey strain. The lamb abortion and arthritis strains caused air sacculitis and the lamb arthritis strain also produced arthritis in turkeys. Strains with no effect were bovine abortion (EBA) and bovine encephalomyelitis.

Cultural studies show that chlamydiae can be isolated from the pericardial sac of orally-inoculated turkeys up to 54 days after detection of bacteremia.\textsuperscript{138} Uninoculated turkeys, when put in contact with the latently-infected ones, became infected with chlamydiae. Even though it is suspected that most chlamydial outbreaks in turkeys are the result of exposure to feral (migratory) birds, Pages's findings suggest
that a carrier state could be important in the epidemiology of this disease.\textsuperscript{74,138}

None of the papers cited described ultrastructural lesions associated with early stages of infection nor did they completely document the progression of lesions histologically. The lesions associated with subclinical infection and clinicopathologic changes also were not studied.
Part I. RESPIRATORY AND PERICARDIAL PATHOLOGY OF TURKEYS INFECTED WITH AVIAN OR MAMMALIAN STRAINS OF CHLAMYDIA PSITTACI
Respiratory and Pericardial Pathology of Turkeys Infected with Avian or Mammalian Strains of *Chlamydia psittaci*

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Running title: Chlamydiosis in turkeys

This paper is part of a dissertation submitted by the senior author in partial fulfillment of the requirements for the PhD degree in Veterinary Pathology, Iowa State University, Ames, IA.
ABSTRACT

Three groups of turkeys were inoculated with strains of \textit{C. psittaci} (B577, VS1, TT3) from different restriction endonuclease (RE) groups. Turkeys were necropsied at 15 times through post-inoculation day (PID) 70. Birds infected with the TT3 strain were lethargic and had decreased body weight. Dyspnea after forced exercise was in VS1-infected turkeys. Pericarditis was the most severe lesion in TT3-infected birds. Airsacculitis and bronchopneumonia were the most severe lesions in VS1-infected turkeys. Lateral nasal adenitis was in both VS1- and TT3-infected birds. Only mild peribronchial pneumonia was in B577-infected turkeys. Chlamydial antigen, identified by light microscopy using an immunoperoxidase technique, was seen from PID 9-50 in the lateral nasal gland and at earlier times in other tissue from VS1- and TT3-infected turkeys. No chlamydial antigen was detected in tissue from B577-infected birds. These studies showed that chlamydial strains from different RE groups are associated with distinct disease syndromes in turkeys.
INTRODUCTION

Some strains of *Chlamydia psittaci* are associated with distinct syndromes in turkeys.\(^8,9,15,23,24,25,26\) The comparability of results and conclusions from these studies is diminished by differences in chlamydial propagation techniques, numbers of chlamydiae given, route of inoculation, and times from inoculation to necropsy. Use of different turkey strains and variation in housing conditions also complicates comparison of findings.

Historically, chlamydiae have been classified on the basis of their virulence patterns in laboratory animals or animals in natural outbreaks.\(^23\) Chlamydiae used in turkey pathogenicity studies were classified according to virulence for turkeys in natural outbreaks, virulence for parrots and tropism for ovine/bovine reproductive organs or joints. Although these classification schemes are useful, they are inexact and technically difficult to reproduce.

Precise classification of strains of *Chlamydia psittaci* can be done by restriction endonuclease (RE) analysis.\(^3,4\) Avian strains can be separated into 2 groups (virulent turkey or psittacine); each is distinct from mammalian groups. To test our hypothesis that specific RE groups are associated with specific pathologic syndromes, we compared, in turkeys,
the respiratory and pericardial lesions caused by a mammalian strain and strains from the avian groups.
MATERIALS AND METHODS

Four groups of 20 twelve-week-old, commercially raised, unvaccinated, broad-breasted white turkeys were maintained in isolation, in individual groups, until necropsy.

Aerobic bacteriologic cultures of the cloaca, done 4 days before inoculation of chlamydiae, were taken from 12 birds randomly selected from each group. *E. coli* isolates were serotyped.

Serum samples from turkeys from each group (8 birds at end of study) were tested for antibodies against *Mycoplasma gallisepticum*, *M. synoviae*, and *M. meleagris* whole cell antigen preparations using the rapid serum plate test. Samples were confirmed as positive or negative by a dot-blotting technique (Donna Cummins, National Veterinary Services Laboratory, Ames, IA).

Chlamydiae were grown in Vero cells, centrifuged at low speed to remove cell debris and then concentrated by high speed centrifugation. Chlamydial elementary bodies (EB) were resuspended as a 1:10 (v/v) suspension of EB and sucrose phosphate buffer and frozen at -80°C. On the inoculation day, EB were thawed and diluted with phosphate buffered saline (PBS). Turkeys were placed in right lateral recumbency and inoculated intratracheally with 1 ml of PBS containing
approximately 10,000 elementary bodies. Chlamydia strains used were TT3 (Texas turkey 3, virulent turkey group), V51 (psittacine group) and B577 (ovine abortion agent). One group of turkeys was given only PBS and served as controls. All turkeys were clinically normal after inoculation and were observed daily until necropsy.

One turkey from each group was killed with sodium pentobarbital (Pentobarbital sodium solution, Fort Dodge Laboratories, Fort Dodge, IA) given IV and necropsied at 1, 2, 3, 4, 7, 9, 11, 14, 17, 24, 31, 39, 50, 60 and 70 days post inoculation (PID). All organ systems were examined grossly and tissue samples were fixed in 10% neutral buffered formalin. A 1 cm transverse section of nasal tissue immediately rostral to the eyes was collected. Tracheal samples were taken caudal to the inoculation site. One-half centimeter, transverse sections of each lung were taken at the level of the origin of the intrapulmonary primary bronchus (i.e. where the extrapulmonary primary bronchus enters the pulmonary parenchyma). Both abdominal air sacs were dissected free, placed in tissue cassettes and immersed in formalin. Pericardium was fixed in a similar manner. The trachea and lungs were pre-fixed within 2 minutes of death by intratracheal perfusion of 50 ml of 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4).

Mycoplasma cultures were done on air sac swabs taken PID
60 and 70 from 16 turkeys (each group sampled; 8 birds sampled on PID 70 were extras, not necropsied). Specimens were transferred to broth and agar media and incubated at 37 C in 5% CO2 for at least 14 days as described by Yoder.29

Lung, air sac and pericardium were collected on PI days 4, 7, 14, 31, 39, 50, 60 and 70 and fecal samples were collected from the large intestine on PI days 2, 7, 9, 11, 17, 31, 39, 50, 60 and 70 for chlamydial isolation. All samples were frozen at -80°C before isolations were attempted.

Samples were thawed and added to 4 ml of Eagle's minimum essential medium to a dilution of 1:80. This mixture was centrifuged at 650 X g for 10 minutes. The center layer of the centrifugate was inoculated into a 24-well microtiter plate at a quantity of 0.25 ml/well. The wells contained confluent layers of Vero cells. The plates were centrifuged for 1 hour at 900 X g at 25°C. The inoculum was removed and replaced with 1 ml of Eagle's minimum essential medium with Earle's balanced salts, 20 mmol/L of Hepes 5% fetal bovine serum, 5.4 mg/L of glucose, 292 mg/L of glutamine, 2 ug/ml of amphotericin B, 200 ug/ml of gentamicin sulfate and 0.5 ug/ml of cyclohexamide. Vero cells were fixed with 50% acetone/50% methyl alcohol at 3 and 6 days after inoculation and stained with a group-reacting monoclonal antibody (B577-D1). Two hundred microliters of a 1:500 dilution of antibody was added per well and reacted with the cells for 30 minutes. The cells
were washed and to each well was added 200 μl of a fluorescein-conjugated antimouse IgG (heavy and light chain specific) (Cooper Biomedical, Inc., Malvern, PA) at a dilution of 1 to 30 for 30 minutes. The cells were washed and examined under an epifluorescence microscope for staining of inclusions.

For light microscopy, samples were processed by standard procedures and embedded in paraffin. Sections were cut at 4 μm and stained with hematoxylin and eosin (HE) or Giminez stain. Lateral nasal gland lesions were scored: 1+ when interstitial infiltrates of inflammatory cells without distortion of glandular architecture were seen; 2+ when inflammatory cell infiltrates were severe enough to compress adjacent ductules; 3+ when 2+ changes plus duct/ductular ectasia and epithelial hyperplasia and intraluminal exudate were seen. Tracheal lesions were scored: 1+ when 1-2 inflammatory cells/high power field (hpf) (average of 4 fields) were seen in the lamina propria; 2+ when 3-5 inflammatory cells/hpf were seen in the lamina propria; 3+ when > 5 inflammatory cells/hpf and/or mucosal epithelial cell hyperplasia were seen. Lung lesions were scored: 1+ when bronchus-associated lymphatic tissue was increased versus control and/or there was hypertrophy of air capillary epithelial cells involving > 10% of the section; 2+ when there was hypertrophy of air capillary epithelial cells involving >
10% of the section and heterophils in > 20% of blood capillaries; 3+ when there were focally-extensive (> 200 um diameter) to diffuse inflammatory cell infiltrates in airways; 4+ when the lesions of 3+ plus necrosis were seen. Air sac lesions were scored: 1+ when there were focal-diffuse inflammatory cell infiltrates in the lamina propria and not > 10% of the membrane thickened; 2+ when there was inflammation and thickening of > 10% of the membrane up to 200 um; 3+ when the air sac was inflamed and > 200 um thick; 4+ when the lesions of 3+ were present and there was necrosis. Pericardial lesions were scored: 1+ when < 10% of the membrane was thickened and had focal-diffuse inflammatory cell infiltrates in the lamina propria; 2+ when > 10% of the membrane was thickened up to 80 um and inflamed; 3+ when > 10% of the membrane was thicker than 80 um and inflamed. Lesion scores from each tissue, for each group, were summed and averaged. Tests for statistically significant differences between means of TT3 and VS1 lesion scores and of B577 and PBS lesions scores were done using Student's t test.

Chlamydial antigen was detected in tissues by an immunoperoxidase technique that used biotinylated secondary antibody and an avidin-biotin-peroxidase complex. Primary antibody was a genus-specific monoclonal antibody (Cultureset® chlamydia identification kit, Ortho Diagnostic Systems, Inc., Raritan, NJ), biotinylated anti-mouse IgG and
the avidin-biotin-peroxidase complex are also commercially available (Vectastain (TM) ABC kit, Vector Laboratories, Inc., Burlingame, CA). A modified version of the technique reported by Moore and Petrak 22 was used. After incubation in the primary antibody for 3 hours at 37°C, the sections were rinsed in phosphate-buffered saline (PBS) and incubated in the secondary antibody for 1 hr at room temperature. The remainder of the procedure is described in the Vectastain (TM) ABC kit instructions under "Staining procedure for paraffin sections". Sections of chlamydia-infected tissue (as determined by culture, histochemistry or electron microscopy) were used to assess the sensitivity and specificity of the procedure. Antigen was detected in sections of B577-infected, V51-infected and TT3-infected tissues as well as in tissues infected with unknown chlamydial strains. When the primary antibody was replaced with PBS or normal mouse serum, or when the secondary antibody was replaced with PBS, no staining was seen. Areas in tissue sections with the highest antigen density were scored as 0 if they had no cells containing antigen, 1 if they had 1-3 antigen positive cells per high power field (hpf) (400X), 2 if they had 4-8 antigen positive cells per hpf and 3 if they had greater than 8 antigen positive cells per hpf.
RESULTS

Clinical Findings

Clinical signs were seen in the TT3-infected group on PID 4–24; turkeys were lethargic and had droopy wings. Birds in this group had blepharospasm and decreased responses to auditory stimuli on PID 9–11. Two TT3-infected turkeys died on PID 11. Yellow feces were on the floor of their room on PID 11 and from this day through PID 50 these birds were judged to be 60–80% of the body weight of birds in the other groups.

Birds in the VS1 group had mild dyspnea (after forced exercise) on PID 4–11 and on PID 9 they were slightly lethargic. Control and B577-infected turkeys were normal throughout the study.

Bacteriology and Serology

Chlamydiae were isolated from tissue samples until PID 14 and from fecal samples until PID 7. Chlamydial antigen was detected, by immunoperoxidase staining, in all tissue types (Figs. 1–6).

Serum, taken PID 70 from 8 turkeys, did not have evidence of antibodies to mycoplasma. No mycoplasma were isolated from
any turkey.

Cloacal samples from 35 birds contained *E. coli*. Serotyping did not identify isolates commonly associated with disease in turkeys.

**Gross Pathology**

Lesions were seen first in VS1-infected birds. On PID 2 there was mild thickening and translucency of the right abdominal air sac. Air sac lesions became most severe during PID 7-11 and then progressively abated; the severest lesions were bilateral, marked thickenings caused by large amounts of fibrin.

Pericarditis was first seen in VS1-infected birds on PID 7 when there were a few flecks of fibrin on the epicardium. On PID 9 the epicardium was covered with a web-like layer of exudate and there were adhesions between visceral and parietal pericardium. No pericardial lesions were noted after PID 9 except on PID 17 when 1 ml of watery, cloudy fluid with a small amount of fibrin was seen in the pericardial space.

Pneumonia, characterized by a 0.2-0.5 cm brown-gray area of consolidation, was only in the right lung of VS1-infected birds and only from PID 2-11. No foci of pneumonia were seen in TT3-infected turkeys.

Pericarditis, the most severe lesion in TT3-infected
turkeys, was first seen on PID 4; there was 1 ml of watery fluid with flecks of fibrin in the pericardial space. Lesions were most severe PID 9-17 when there was severe thickening of the pericardium (up to 1 cm) due to edema and accumulation of fibrin. Pericarditis, with numerous fibrous adhesions between visceral and parietal pericardium, was still noticeable on PID 70.

Airsacculitis was not as severe in TT3-infected versus VS1-infected turkeys. At 24hr PI there was focal translucency of the right abdominal air sac. At PID 4 there was diffuse translucency of the right abdominal and interclavicular air sacs. The most severe air sac lesions were seen on PID 14 and 17 when there was mild thickening (0.2 cm) and opacity due to edema and occasional foci of fibrin. No air sac lesions were seen in TT3-infected turkeys after PID 39 except focal fibrosis of the right abdominal air sac from the turkey necropsied on PID 50.

Changes in turkeys infected with the B577 strain were minimal multifocal thickening and translucency of the right thoracic or abdominal air sacs on PID 4-11 and five 0.2-0.3 cm fibrous plaques on the epicardium on PID 14.

There were no gross lesions of the tracheal or nasal mucosa in any turkey.
Histopathology

Lateral nasal glands

Significant lesions were only in VS1- and TT3-infected turkeys (Fig. 7); they developed later than those in other organs. Interstitial infiltrates of heterophils and plasma cells were seen on PID 7 and 11 in TT3 and VS1-infected birds, respectively. The severity of inflammation increased through PID 31 in TT3-infected turkeys and through PID 17 in VS1-infected birds. The most prominent lesions were: ductular ectasia with epithelial hyperplasia and squamous metaplasia, intraductular exudate composed of heterophils, macrophages and sloughed epithelial cells mixed with protein, and interstitial fibrosis with lymphocytic nodules and infiltrates of plasma cells, heterophils and macrophages (Fig. 8,9). Fibrosis was not a prominent lesion in VS1-infected birds. Chlamydial antigen was in macrophages and epithelial cells in intraductular exudate, in interstitial macrophages and occasionally in ductular epithelial cells (Table 1; Figs. 1,2). On PID 70, in turkeys from both groups, there were mild interstitial infiltrates of plasma cells and heterophils; TT3-infected birds also had mild interstitial fibrosis and few dilated ductules.
Trachea

Lesions were seen consistently only in VS1- and TT3-infected turkeys (Fig. 7) from PID 2-17 and 3-14, respectively. Both groups had similar lesions that initially were characterized by mild infiltrates of heterophils in the lamina propria. The severest change was a moderate infiltrate of heterophils, plasma cells and macrophages in the lamina propria, moderate diffuse atrophy of mucosal glands and mild, multifocal hyperplasia of mucosal epithelial cells. Chlamydial antigen was in submucosal glandular epithelial cells, macrophages and occasional endothelial cells.

Lungs

Lesions graded as 1+ and 2+ were frequently in lungs from each group (including controls).

The most severe lesions were in the right lung of VS1-infected turkeys from PID 2-14 (Fig. 7). They had focally-extensive bronchopneumonia that was characterized by edema and dense infiltrates of lymphocytes, heterophils and macrophages. Parabronchi and atria were dilated, lined by hypertrophied and hyperplastic epithelium, and filled with fibrin and small numbers of macrophages and heterophils (Fig. 10). Chlamydial antigen was in the cytoplasm of macrophages in the submucosa of bronchi on PID 1. In inflamed areas, most antigen positive cells were macrophages (Fig. 3). Rarely, antigen was in the
cytoplasm of bronchial epithelial cells.

Lesions in the left lung of VS1-infected turkeys resembled those in the right lung but were centered around the IPB, were not as extensive and were of shorter duration. There were no significant histopathological differences between the right and left lungs of TT3-infected turkeys. Lesions were never as severe as those in VS1-infected birds. Two birds had mild, focal bronchitis, but none had bronchopneumonia. Several samples of lung contained foci of acute inflammation involving up to 6 lobules. These were characterized by edema and occasional heterophils in air capillary lumens, hypertrophy of ACEC and sequestered heterophils and hypertrophied endothelial cells in blood capillaries (Fig. 11). Antigen positive cells were most commonly in walls of air capillaries (Fig. 4) but we could not determine if these cells were epithelial, endothelial or intraluminal. Antigen positive macrophages were in occasional small inflammatory foci.

Lesions in B577-infected birds resembled those in VS1-infected turkeys but were less severe, seen at fewer necropsy times and only in the right lung (except PID 4). These changes were centered around the junction of the IPB with a secondary bronchus and extended only a short distance into the adjacent connective tissue or parenchyma. On PID 17 and 50 there was mycotic bronchopneumonia in the right lung.
Abdominal air sacs

Air sacs with 1+ lesions had mild, multifocal inflammation and could not be distinguished from controls.

Airsacculitis was the predominant lesion in VS1-infected birds (Fig. 7). These changes were prominent 2-50 days PI and progressed from marked edema with infiltrates of heterophils to fibrosis with numerous lymphocytic nodules with germinal centers. Vasculitis, characterized by endothelial cell hypertrophy and hyperplasia, intramural edema and infiltrates of heterophils and/or macrophages in and around vessel walls, was seen on PID 2-14 (Fig. 12). The severest vascular inflammation was on PID 4-9; the days when the most fibrin was seen on the epithelial surface of the air sacs. There was marked epithelial cell hyperplasia on PID 4-17.

Air sac lesions in TT3-infected turkeys were not as severe as those seen in VS1-infected birds. The most prominent lesions were on PID 14 when there was moderate diffuse thickening of the air sacs due to edema and infiltrates of plasma cells, macrophages and a few heterophils in the lamina propria. Vascular lesions were mild and characterized by endothelial cell hypertrophy and perivascular infiltrates of plasma cells and lymphocytes. There was mild hyperplasia of epithelial cells on PID 17-39.

High numbers of chlamydial antigen-positive cells were in
air sacs from VS1 and TT3-infected turkeys. Antigen was most common in macrophages in the lamina propria (Fig. 5) and in exudate on the respiratory surface. Small numbers of air sac epithelial cells in several specimens contained antigen. On PID 14 the left air sac from a TT3-infected turkey had several antigen positive mesothelial cells. Chlamydial antigen was rarely in vascular endothelial cells.

Pericardium

Pericarditis was the predominant lesion in TT3-infected turkeys (Fig. 7). The pericardium was markedly thickened due to edema, dilation of lymphatics and infiltration of inflammatory cells. Macrophages, plasma cells and heterophils frequently contained chlamydial antigen (Fig. 6). Vasculitis, involving most small veins, was seen on PID 4-14 (Fig. 12). Large amounts of fibrin covered the pericardial surface on PID 9-17. On subsequent PID there were decreased amounts fibrin and increased numbers of large fibrous papillae and adhesions between visceral and parietal pericardium. Lymphocytic nodules with germinal centers were seen on PID 24 – 60.

Pericardial lesions in VS1-infected turkeys were less severe and seen at fewer necropsy times than those in TT3-infected birds. The most severe lesions were on PID 7 and 9 when there was thickening of the membrane due to edema, dilation of lymphatics, hyperplasia of pericardial lining
cells (PLC) and infiltrates of plasma cells, heterophils and macrophages. Only a few macrophages contained chlamydial antigen. Fibrin, in small amounts, was seen only on PID 9. Vascular lesions were not as severe or as disseminated as those in TT3-infected birds. On PID 17 the pericardium was characterized by diffuse hyperplasia of PLC with formation of numerous small papillae. Subjacent to PLC was a zone containing a dense infiltrate of plasma cells and small lymphocytes. After PID 17 the only lesions were occasional perivascular infiltrates of lymphocytes and moderate numbers of fibrous papillary projections.

The only lesion in the pericardium of a B577-infected turkey was on PID 14 when there were numerous papillary projections similar to, but smaller than, those in VS1-infected birds.

Control turkeys frequently had papillary projections from the pericardial surface but usually less than 3 per section and if multiple were localized to one region of the pericardium. They were composed of PLC and generally did not have fibrous stalks or infiltrates of inflammatory cells.
Fig. 1  Immunoperoxidase localization of chlamydial antigen (TT3), lateral nasal gland, PID 14. Note large amounts of antigen in interstitial macrophages and intraductular exudate.

Fig. 2  Immunoperoxidase localization of chlamydial antigen (TT3), lateral nasal gland, PID 14. Note antigen positive cells in dilated interlobular duct.

Fig. 3  Immunoperoxidase localization of chlamydial antigen (VS1), lung, PID 2. Note antigen positive macrophages in atrium.

Fig. 4  Immunoperoxidase localization of chlamydial antigen (TT3), lung, PID 9. Note antigen positive cells in air capillary walls.

Fig. 5  Immunoperoxidase localization of chlamydial antigen (VS1), right abdominal air sac, PID 4. Note numerous antigen positive macrophages in the lamina propria.

Fig. 6  Immunoperoxidase localization of chlamydial antigen (TT3), pericardium, PID 9. Note antigen positive macrophages in exudate and superficial lamina propria. No antigen positive cells in inflamed vein.
Fig. 7 Bar graph of lesion scores from controls (PBS) and turkeys infected with chlamydiae from different RE groups. Each bar = mean lesion score, n=15 birds per exposure group. * = significantly different from VS1, p < 0.05. (Criteria for lesion scoring described in materials and methods)
Left Air Sac

Right Air Sac

Left Lung

Right Lung

Lateral Nasal Gland

Pericardium

Trachea
Fig. 8  Lateral nasal gland from a control turkey. HE.

Fig. 9  Lateral nasal gland from a TT3-infected turkey (PID 31). Note dilated and exudate-filled ductules and interstitial fibrosis. HE.
Fig. 10  Right lung from a VS1-infected turkey (PID 2). Note obliteration of parenchyma by dense inflammatory cell infiltrates and dilated and fibrin-filled parabronchus.
Fig. 11  Right lung from a TT3-infected turkey (PID 9). Note hypertrophy of air capillary epithelial and blood capillary endothelial cells.
Fig. 12  Pericardial vein from a TT3-infected turkey (PID 9). Note endothelial cell hypertrophy and intramural and perivascular edema and infiltrates of inflammatory cells. Similar changes were in blood vessels of air sacs from VS1-infected birds.
Table 1. Immunoperoxidase detection of chlamydial antigen

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<th>Left Lung VS1</th>
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<th>Right Air Sac VS1</th>
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PID = Post inoculation day
- = Negative
1 = 1-3 Antigen positive cells (apc) per high power field (hpf)
2 = 4-8 apc/hpf
3 = >8 apc/hpf
ND = Not done
DISCUSSION

Chlamydial strains in the same RE group cause similar lesions in turkeys. These lesions are distinct from those caused by strains in other RE groups. This premise is supported by studies in which chlamydial strains related to ours were used. Pericarditis was the most prominent lesion caused by a Texas turkey strain and by NJ1 and SCT1 chlamydial serotypes (serotypes grouped by restriction endonuclease analysis with TT3) in turkeys. In contrast, a low incidence of pericarditis was seen in turkeys infected with psittacine chlamydial isolates. One of these isolates (6BC) cross reacts with monoclonal antibodies to VS1 and they have similar RE analysis patterns.

Airsacculitis is severe in turkeys infected with psittacine isolates but not in turkeys infected with strains highly virulent for turkeys. Although airsacculitis was in most of our TT3-infected turkeys, it was seen later and was much less severe than air sac lesions in VS1-infected birds. Other investigators inconsistently saw airsacculitis in turkeys infected with NJ1, SCT1 and Texas turkey chlamydial strains. In contrast, there was a high incidence of airsacculitis in turkeys infected with chlamydial strains (including 6BC) of psittacine origin. Strains SCT1 and NJ1
cross react with monoclonal antibodies to TT3 and all 3 strains have similar RE patterns. The VS1 strain similarly cross reacts and groups with strain 6BC.

Differences in severity of inflammation in the air sacs and pericardium of TT3- (and also VS1) infected turkeys could be explained by differences in endothelial cell populations in these organs. Organ-specific endothelial cells have been identified in lymphoid and synovial tissues. The more severe vasculitis seen in the pericardium could be the result of chlamydial antigen attachment to receptors on pericardial endothelium that are not present on air sac endothelial cells. Attachment of antibodies and complement activation would cause inflammation of these vessels. The endothelial cells would not necessarily have to be infected by the bacterium; chlamydial antigen could originate from plasma membrane-associated antigen released from chlamydia-infected macrophages in other organs (i.e. spleen). At least 2 studies have shown that chlamydia infected cells release chlamydia-derived antigen that can be incorporated into plasma membranes of neighboring cells. Low levels of this antigen, alteration by antibody binding or subsequent inflammation, or lack of binding to the primary antibody used in our procedure could explain the only rare occurrence of antigen positive endothelial cells in our immunoperoxidase studies.

It is more difficult to explain why there were similar
numbers of antigen positive cells, but different degrees of inflammation, in the pericardium and air sacs of TT3-infected turkeys. The presence of small numbers of macrophages in normal air sacs may be the result of expression of low levels of endothelial-leukocyte adhesion molecule 1 (E-LAM 1) on air sac endothelium. Increased expression of E-LAM 1, caused by high levels of circulating interleukin 1 (i.e. from activated macrophages in the spleen or other organs), could result in infiltration of higher than usual numbers of macrophages into air sacs. Previous or subsequent infection of these cells would account for the antigen positive cells seen by immunoperoxidase staining.

Bronchopneumonia is characteristic of VS1-infected turkeys but not of turkeys infected with TT3 and related strains. No SCT1-infected turkeys had histologic evidence of pneumonia. There was a low incidence of pneumonia in older (>10 weeks) birds infected with the Texas turkey strain. The lesions seen could have been a reaction to the amnioallantoic fluid used in the inoculum.

We could not detect, by immunohistochemistry, large numbers of chlamydiae in sections of lung from TT3-infected turkeys (except on PID 9). These findings differ from those of Page's cultural studies. He found extensive multiplication of chlamydiae in the lungs of turkeys infected with the NJ1 strain (related to TT3) 24 hours post
Inoculation. These differences might be related to the types of inocula used. We used elementary bodies free of extraneous protein. Page used beef heart broth containing either ground mouse spleen and liver or yolk sac. Viable cells in this material could have allowed chlamydial replication.

Our findings indicate that mammalian abortion strains of C. psittaci are nonpathogenic for turkeys. Page found only multifocal airsacculitis on gross examination of turkeys infected with an ovine abortion strain and no lesions in turkeys infected with EBA strain chlamydiae (originally isolated from an aborted bovine fetus). Monoclonal antibodies that bind to the EBA strain cross react with the B577 strain. In addition, the RE analysis patterns of these strains are similar. The inability to reisolate the ovine chlamydia suggests this strain does not multiply or persist in turkeys. Our findings are also supported by Johnson and Grimes who were unable to detect seroconversion or isolate chlamydiae from tissue suspensions or cloacal swabs from wild birds inoculated with the B577 strain.

Our inability to reisolate or detect B577 strain chlamydiae in tissue sections by immunoperoxidase staining suggests the mild pneumonia seen on PID 2–14 was not due to chlamydial infection. We interpreted pneumonic lesions to represent a reaction to nonviable foreign protein, similar to that in rat lungs exposed to ovalbumin. The inability of the
B577 strain to survive or multiply could be related to interferon levels or inappropriate amino acid concentrations in turkey respiratory tissue. The intracellular growth of *C. psittaci* is reversibly inhibited by gamma interferon and this inhibition is concentration dependent.\(^{13}\) Amino acid requirements differed for 4 *C. psittaci* isolates in tissue culture. One of these isolates, EAE (enzootic abortion of ewes), was unique in its requirement for tyrosine.\(^{1}\)

Infected lateral nasal glands could be an important source of aerosolized chlamydiae since these glands are the main source of moisture for the nasal mucosa.\(^{6}\) Late and long term infection of these glands, as indicated by the immunohistochemical findings, implicates them as potential reservoirs for chlamydiae. In addition, these findings suggest examination of oronasal fluids (vs. the common practice of collecting cloacal material) may be useful in detecting infected birds. Additional studies are required to test these hypotheses.

Freezing the samples before isolation attempts were done undoubtedly influenced our isolation success rate. Dilution of the samples, small sample size (i.e. air sacs and pericardium) and use of low centrifugation temperatures also would impede isolation attempts.

Chlamydial strain-specific and tissue specific receptors may be involved in the RE group-related differences
in tissue tropisms. The ability of myeloid cells and monocytes to bind *C. trachomatis* (L2 serovar) is dependent on acquisition of specific antigens during maturation. Beuth, et. al., have shown that adherence to pulmonary tissue by *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* is dependent on specific bacterial surface lectin receptors. Furthermore, they suggest these receptors are strain specific. Limited studies of lectin-inhibited binding of strains of *C. trachomatis* to tissue culture cells have given conflicting results but suggest chlamydiae have strain-specific lectin receptors.
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Part II. INHIBITION OF ENDOSONAL ACIDIFICATION BY
CHLAMYDIAL ELEMENTARY BODIES
Inhibition of endosomal acidification by chlamydial elementary bodies

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Running title: EB endosomal acidification

This paper is part of a dissertation submitted by the senior author in partial fulfillment of the requirements for the PhD degree in Veterinary Pathology, Iowa State University, Ames, IA.
ABSTRACT

The hypothesis that chlamydiae inhibit endosomal acidification was tested by infecting J774 cells with the B577 strain of Chlamydia psittaci and assaying for endosomal acidification. Electron microscopy, immunogold techniques, and image analysis were used to determine the extent of DAMP labeling (which is proportional to acidity) in J774 cellular endosomes containing either live or heat-inactivated elementary bodies. Live, but not heat-inactivated, elementary bodies inhibited endosomal DAMP accumulation as early as 30 seconds, and up to 30 minutes, after infection of J774 cells. Lysosomes were seen fused with endosomes containing heat-killed, but not live, chlamydiae. These findings suggest that inhibition of endosomal acidification is a mechanism whereby chlamydiae inhibit phagolysosomal fusion and evade antigen processing.
INTRODUCTION

Chlamydiae are pathogenic intracellular bacteria that cause acute or chronic recurrent disease syndromes associated with persistent infections. In the protected environment of a host cell endosome chlamydiae evade the humoral immune response, resist antigen processing, and elaborate antigens that induce immune responses that are detrimental to their host.\textsuperscript{12,14,28,43} Although the morphological changes in the chlamydial developmental cycle, i.e. from the elementary body (EB) to the metabolically active reticulate body, and back again, are well documented, the mechanisms by which these forms enter and survive within the cell are unclear. Knowledge of the mechanism by which chlamydia-containing endosomes resist fusion with lysosomes would be particularly useful because it would help understand how to manipulate the organism for development of an effective vaccine.

Acidification of endosomes is a fundamental cellular process necessary for sorting, routing, and degradation of endocytosed material, all of which are necessary for effective antigen processing.\textsuperscript{19,26} There are two types of nonlysosomal acidic endosomes, "early" and "late", with average pHs of 6.2 and 5.3, respectively. Only late endosomes transfer material to lysosomes.\textsuperscript{29} Neutralization of endosomal pH blocks antigen processing and transfer of ligands from endosomes to lysosomes.
suggesting acidification also is necessary for endosomal fusion.\textsuperscript{4,7,19,38,39} Fusion of digestive vacuoles (DV) with lysosomes occurs only after DV acidification in \textit{Paramecium caudatum}.\textsuperscript{1} Because other organisms that inhibit phagolysosomal fusion (i.e. \textit{Toxoplasma gondii}, \textit{Glugea hertwigi}, \textit{Legionella pneumophila} and \textit{Mycobacterium tuberculosis}) also reside in nonacidified endosomes, and because neutralization of endosomal pH causes inhibition of fusion of late endosomes with lysosomes and disrupts antigen processing, we hypothesize that chlamydiae inhibit acidification of their endosomes.\textsuperscript{4,7,18,19,20,40,42}

The purpose of this study is to determine: 1) if chlamydiae inhibit endosomal acidification in a mouse macrophage cell line, 2) when in the endocytic process the acidification block occurs, and 3) if heat-killed chlamydial elementary bodies (EBs) also inhibit acidification.
MATERIALS AND METHODS

An ovine abortion (B577) strain of C. psittaci was used in all experiments. This strain was chosen because of its low infectivity (versus avian strains) for humans; the risk of infection was increased because numerous, rapid manipulations were required to do these experiments.

Chlamydiae were grown in Vero cells, centrifuged at low speed to remove cell debris and then concentrated by high-speed centrifugation.\(^2\) Chlamydial elementary bodies were resuspended as a 1:10 suspension of EBs and sucrose phosphate buffer and frozen, in aliquots, at -80°C. The day before inoculation EBs were thawed, diluted in phosphate-buffered saline (PBS), washed once by high-speed centrifugation, and resuspended in PBS (at an average multiplicity of infection of 2.5 EBs per cell). An aliquot of EBs was killed by incubating at 60°C for 1 hour. All EB aliquots were stored overnight at 4°C before use.

Preliminary evidence that chlamydiae reside in mildly acidified or nonacidified endosomes was gained from experiments in which the acidotropic amine chloroquine was used. Chloroquine accumulates in acidic cellular compartments. Vero cells were used as hosts. Cells were grown to confluency in 75 cm\(^2\) tissue culture flasks (Falcon - Becton Dickinson and Co., Lincoln Park, NJ) and inoculated
with 10⁵ live EBs in 1 ml PBS. Control flasks were given only PBS. At 24 hours post inoculation culture medium was removed and replaced with 20 ml of medium containing 1 mM chloroquine. After 6 hours the cells were scraped free from the flask, centrifuged into a pellet, and resuspended in 20 ml of cold 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer. After 20 minutes they were washed 3 times, by alternate centrifugation and resuspension, in 0.1 M Na cacodylate buffer. The last pellet was resuspended in molten agar, cooled at 4°C until gelatinized, cut into 2 mm cubes and processed routinely for transmission electron microscopy.

To gain more precise information about the acidity of chlamydia-containing endosomes, when inhibition of acidification occurred, and if it could be mediated by heat-inactivated EBs, J774 cells (J774A.1, BALB/c mouse macrophage cell line, obtained from the American Type Culture Collection, Rockville, MD) were infected with live or heat-killed EBs for various times followed by exposure to DAMP (Table 1). Culture plate inserts (Millicell HA, 0.45um, 12mm diameter, Millipore Corporation, Bedford, MA) were placed in 24-well tissue culture plates (Costar, Cambridge, MA), seeded with 75,000 cells (per insert), and cultured, before use, for 5 days in Dulbecco's modified Eagle medium (DMEM) (GIBCO Laboratories, Grand Island, NY) containing 10% fetal bovine serum and 327 mg of glutamine, 27.5 ug amphotericin B, and 10 mg gentamicin
Experimental Design

Insert membranes containing J774 cells were cooled for 1 hour at 4°C before use. Cells were inoculated with 0.2 ml of PBS (4°C) containing an average of 2.5 EBs per cell and maintained at 4°C for 2 hours. Incubation times (0.5, 2, 10, and 30 minutes) were started by replacing the PBS with 0.8 ml of DMEM (37°C). After each incubation DMEM was aspirated, membranes were washed once with PBS (37°C), and inserts were filled with 0.8 ml of DMEM containing 60 uM of 3-(2,4-dinitroanilino)-3'-amino-N-methlydipropylamine (DAMP) (Oxford Biomedical Research, Inc., Oxford, MI). DAMP accumulates, and is retained by fixation, in organelles with an acidic luminal pH. DAMP accumulation is directly proportional to the luminal pH. Cells were incubated with DAMP for 5 minutes (DAMP omitted from 1 set of inserts as controls) at which time inserts were aspirated, washed once with PBS (4°C) and fixed for 15 minutes in 1% glutaraldehyde in Dulbecco's PBS (pH 7.2) (Sigma Chemical Co., St. Louis, MO). Membranes then were washed 3 times in Dulbecco's PBS, incubated 30 minutes in Dulbecco's PBS containing 0.5M NH₄Cl, and again washed 3 times in Dulbecco's PBS. Postfixation was for 1 hour at room
temperature in 1% osmium tetroxide in 100mM Na$_2$HPO$_4$ (pH 7.2) (phosphate buffer) followed by 3 washes in phosphate buffer. Membranes then were incubated for 30 minutes at room temperature in 0.01% tannic acid (in phosphate buffer) followed by 3 washes in the same buffer. They were infiltrated and embedded in epoxy resin (EM-BED 812 kit, Electron Microscopy Sciences, Fort Washington, PA) made up of components in the following proportions: 86 ml of 812, 48 ml of DDSA, 41 ml of NMA and 2.1 ml of DMP-30. Membranes were sectioned at 80 nm and placed on nickel grids (nickel square 200 mesh grid, 3.05mm - Polysciences, Inc. Warrington PA) for immunolabeling.

Immunolabeling Procedure

Sections were etched for 30 minutes (room temperature) in a saturated solution of sodium metaperiodate in water, washed by dipping 10 times in water, blotted dry, and incubated for 1 hour (37°C) in 1% ovalbumin (OA) (Sigma) in Tris buffer (pH 8). Grids then were blotted dry, placed in 0.1% OA in Tris buffer (pH 8) containing 5 ug per ml of anti-DAMP antibody (Oxford) (anti-DAMP antibody omitted from 2nd set of controls), and incubated for 18 hours at 4°C. Ten jets (total of 25 ml) of Tris buffer containing 0.1% OA were used to wash each grid; grids then were placed in a 1:10 solution of gold-
labeled secondary antibody (Auroprobe EM GAM IgG G10 – Amersham Corporation, Arlington Heights, IL), in 0.1% OA in Tris buffer, for 2 hours at 37°C. Grids then were jet-washed (total of 25 ml) with Tris buffer containing 0.1% OA followed by jet-washing (total of 25 ml) in boiled, distilled water, and blotted dry and stained with 2% uranyl acetate and 2.5% Reynolds lead citrate (Electron Microscopy Sciences, Fort Washington, PA). Sections of infected and noninfected cells exposed to DMEM (in place of DAMP) and grids from which the primary antibody was omitted from the labeling procedure were used as controls.

Sampling and Quantitation of Endosomal Acidity

All samples were coded so that the pathologist had no knowledge of the treatment until all quantitative analyses were completed. Samples were examined at 14,500x with a transmission electron microscope. One edge of the membrane was located and the first 3-4 cells containing at least 1 intracellular EB and at least 1 well-defined, colloidal gold-labeled, intracytoplasmic vesicle (not containing an EB) were photographed. "Labeled" was defined as any gold labeling above background levels (as judged by comparing labeling intensity with adjacent mitochondria). Size ranging from 150 nm X 150 nm to 600 nm X 1200 nm, circular to oval shape and
either empty or containing smaller vesicles, laminated membranous whorls, flocculent material or amorphous electron dense material were other criteria for selection as labeled vesicles. These vesicles were designated acidified vesicles (AVs). Two to 4 photographs were taken of each cell to include every EB in the plane of section of the cell. The precise number taken was dictated by the size of the cell, plane of section and the distance between EBs but at least 75% of each cell plane of section was photographed. Photographs were developed and enlarged to 37,700x. On each photograph, to facilitate image analysis, a fine point black marker was used to outline the limiting membranes of each endosome containing EB(s) and each AV.

A Zeiss SEM-IPS image analysis system (Zeiss-Kontron; IBAS version 2.00; Carl Zeiss, Inc., Thornwood, NY) was used to acquire images of the outlined organelles. Photographs were placed on a copy stand and images were captured using a Sony 3 CCD color video camera (Sony, Inc., Montvale, NJ) equipped with a zoom lens and close-up lens. The internal scaling feature of the image analysis software was calibrated for the images under investigation. Colloidal gold particles were automatically counted; particles were differentiated from background by selecting a grey scale pattern, on the computer screen, that best represented the gold particle pattern in the photograph. The area of each organelle also was measured and
gold particles per unit area (GPPU) were calculated. Counts and area measurements were determined from 765 organelles in 191 photographs of 74 cells. GPPUs were converted to pH units, in Table 3, to facilitate comparison with other studies. The pH units were derived from a formula (see below) containing an arbitrarily chosen number; absolute pHs cannot be determined from these data. Because AVs consistently had the highest GPPUs, we assumed that they had the lowest pH; pH 5.0 (the pH of lysosomes and late endocytic vesicles) was used in a modification of a formula to calculate the pHs of EB-containing endosomes. The following formula was used:

\[
\text{pH} = 5.0 - \log \frac{\text{DE}}{\text{DM}}
\]

Where DE = the mean GPPU of EB-containing endosomes in a cell and DM = the mean GPPU of the AVs in the same cell. Analysis of variance was used to test for significant differences between means of pHs.

The reliability of the image analysis system for counting gold particles was determined by comparing the counts of gold particles, done manually and by image analysis, in 87 organelles from 4 cells (total of 11 photographs).
RESULTS

There was 0 – 2% variation in counts of gold particles per unit area when manual and image analysis counting methods were compared (Table 2). This finding establishes the reliability of the image analysis method for determination of gold particle density in organelles.

Chlamydial reticulate bodies were in vesicles of Vero cells incubated in medium with or without chloroquine. Reticulate body-containing vesicles in cells incubated in chloroquine-containing medium were surrounded by numerous, markedly dilated vesicles containing membranous whorls, small vesicles, and amorphous, electron-dense material (Fig. 1). There was no noticeable dilatation of reticulate body-containing vesicles. Uninfected Vero cells exposed to chloroquine contained numerous dilated vesicles similar to those in infected cells.

Cells infected with heat-killed EBs had, at 0.5 min incubation in warmed medium (plus 5 minutes in DAMP), EB-containing endosomes labeled with with a mean GPPU of 13.9. There was no significant change in labeling density of endosomes containing heat-killed EBs over time.

Cells infected with live EBs had, at 0.5 minutes incubation in warmed medium, EB-containing endosomes labeled with a mean GPPU of 3.2. The was no significant change in
labeling density of endosomes containing live EBs over time. The mean of the GPPUs for all of the incubation times was lower for endosomes containing live EBs (mean = 4.2) than for endosomes containing killed EBs (mean = 20.6) (Figs. 2,3); this difference was highly significant (p < 0.01).

Round, electron-dense structures resembling primary lysosomes were seen connected to endosomes containing killed (but never live) EBs. This finding was in occasional cells at 10 minutes incubation but was more common at 30 minutes.
Fig. 1 Chlamydial reticulate bodies in an endosome (arrow) of a Vero cell incubated in medium containing 1 mM chloroquine. The vesicle containing chlamydiae is surrounded by numerous, dilated vesicles containing smaller vesicles, and amorphous, electron dense material (arrowheads).
Fig. 2 J774 cell inoculated with live EBs and incubated for 30 min. in 37°C DMEM. EBs in endosomes (arrowheads) labeled with small numbers of gold particles. Gold-labeled vesicle (AV) containing flocculent material (arrow).
Fig. 3  J774 cell inoculated with heat-killed EBs and incubated for 30 min. in 37°C DMEM. EBs in vesicles (arrowheads) containing numerous gold particles. Vesicles (AVs) labeled with numerous gold particles and containing flocculent material and amorphous, electron dense material.
**TABLE 1.** Numbers of J774 cells examined, at each incubation time, inoculated with live or heat-killed elementary bodies

<table>
<thead>
<tr>
<th>Incubation (min)</th>
<th>Live (Number of Cells Examined)</th>
<th>Killed (Number of Cells Examined)</th>
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<tr>
<td>0.5</td>
<td>12 (4)*</td>
<td>12 (4)</td>
</tr>
<tr>
<td>2</td>
<td>6 (2)</td>
<td>9 (3)</td>
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<tr>
<td>10</td>
<td>7 (2)</td>
<td>6 (2)</td>
</tr>
<tr>
<td>30</td>
<td>13 (4)</td>
<td>9 (3)</td>
</tr>
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</table>

*Numbers in parentheses equals numbers of experiments*
TABLE 2. Comparison of gold particle counting methods for determination of DAMP concentration in organelles

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Counting Method</th>
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<th>Image Analysis</th>
<th>n</th>
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<tr>
<td>Mitochondria</td>
<td></td>
<td>18.5*</td>
<td>18</td>
<td>40</td>
</tr>
<tr>
<td>EB end'</td>
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<td>375</td>
<td>375</td>
<td>17</td>
</tr>
<tr>
<td>AV^</td>
<td></td>
<td>102</td>
<td>100</td>
<td>30</td>
</tr>
</tbody>
</table>

* Mean number of gold particles per unit area
' EB-containing endosome
^ Acidified vesicle
TABLE 3. Estimated pH of endosomes containing live or heat-killed chlamydiae

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
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<th>Killed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>5.8</td>
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<tr>
<td>10</td>
<td>5.7</td>
<td>5.2</td>
</tr>
<tr>
<td>30</td>
<td>5.8</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Mean ± SE\(^1\)  
5.7 ± 0.05  
5.1 ± 0.05

Difference ± SE  
0.60 ± 0.07

\(^1\) SE obtained from pooled estimate of error variance computed from a 2-way ANOVA.
DISCUSSION

The inhibition of endosomal acidification by chlamydiae and other intracellular microbes indicates that this is a common mechanism by which some intracellular pathogens protect themselves from degradation and cause persistent infection. A nonacidified (or mildly acidified) intraendosomal environment would protect the organism from endosomal acid hydrolases and inhibit fusion of the endosome with lysosomes.\(^4,10,11,25\) Although some cell-free assays suggest that fusion of endosomes is independent of internal acid pH, it is questionable whether these models accurately recreate the process of fusion of late (vs. early) endosomes with lysosomes.\(^5,8,9,36\) Almost all of these models use early endosomal preparations; early endosomes are mildly acidic and fuse with one another but not with lysosomes.\(^29,37\)

The marked differences in magnitude of gold labeling of endosomes containing live versus heat-killed EBs, as soon as 0.5 minutes of incubation, suggests that the acidification block is initiated when the EB first attaches to the cell. This hypothesis is supported by other studies that demonstrated: 1) differences in the protein content of membranes from endosomes containing either live or heat-killed EBs, 2) similarities in protein content between EB endosomal membranes and the plasma membrane of the host cell, 3) that
isolated EB cell envelopes, like live EBs, inhibit phagolysosomal fusion, and 4) that protein synthesis by the EB is not required for the initial stages of its intracellular survival.\textsuperscript{12,14,44} In addition, our finding of mild acidification of endosomes containing live EBs suggests that proton pumps are present in the membrane of these endosomes. If so, EBs would require a means for immediate initiation of the acidification block (i.e. recruitment of a molecule(s) on the host cell plasma membrane). Our results would be difficult to explain if EBs inhibited endosomal acidification by blocking fusion of their endosomes with putative proton pump-containing vesicles (the mechanism proposed for inhibition of endosomal acidification by toxoplasma).\textsuperscript{22}

There are at least 2 mechanisms (besides exclusion of proton pumps) by which EBs could inhibit endosomal acidification: 1) by interfering with Cl\textsuperscript{−} conductance into the endosome or 2) by recruitment of additional Na\textsuperscript{+},K\textsuperscript{+}-ATPases into the endosomal membrane.\textsuperscript{3,6,15,16} Interference with chloride conductance would be ruled out because chloride channels are colocalized with proton pumps in the endosomal membrane; they probably would be delivered to the EB endosome with the proton pumps.\textsuperscript{3} Na\textsuperscript{+},K\textsuperscript{+}-ATPases are present in the plasma membrane and therefore are in a position to be recruited by attaching EBs.\textsuperscript{21,35} Allowing the usual number of, or incorporation of additional, Na\textsuperscript{+},K\textsuperscript{+}-ATPases into the EB endosomal membrane (in a
fashion that did not affect ion translocation) would limit or block endosomal acidification. Wheat germ agglutinin, which binds to Na⁺-K⁺ pumps, inhibits binding of C. psittaci EBs to several cell lines. That EBs potentially could attach to ATPases without inhibiting their function is demonstrated by the lack of effect of bound monoclonal antibody on the internalization and function of epidermal growth factor receptors. Inhibition of the removal of Na⁺,K⁺-ATPases from the EB endosomal membrane, because they are attached to adhesins on EBs, would block endosomal acidification, "receptor"-adhesin uncoupling, and routing to lysosomes.

When gold particle labeling densities are converted to pH units (Table 3) the values closely agree with those from studies with Legionella pneumophila. Mild acidification of endosomes containing live chlamydial EBs or Legionella bacilli suggests that exposure to low levels of acidity is beneficial to the organism. Acidification of endosomes containing bacteria may induce expression of genes necessary for growth and reproduction of the organisms and for maintenance of resistance to the host. Mild endosomal acidification also may facilitate intraendosomal cysteine transport; cysteine could reduce disulfide bonds in the EB major outer membrane protein which would allow ATP access to an ATPase on the chlamydial cytoplasmic membrane. Hydrolysis of ATP (to
establish an electrochemical gradient for nutrient transport) is important in the initial stages of infection of host cells by chlamydiae.\textsuperscript{32}

The inefficacy of bacterins in preventing chlamydial infection suggests that modified, live organisms are needed to induce an effective immune response. Our system for determination of the presence of endosomal acidity is ideally suited for the screening of bacterin candidates; properly modified organisms should allow endosomal acidification. Our system also would be useful in the determination of the molecule(s) on the EB surface that is important in inhibition of endosomal acidification. Deletion of the chlamydial gene that codes for this putative molecule is a logical step in antichlamydial vaccine development.
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RESULTS OF THE FIRST STUDY SHOWED THAT STRAINS OF *C. psittaci* FROM DIFFERENT RE GROUPS CAUSED DISTINCT DISEASE SYNDROMES IN TURKEYS. THESE FINDINGS SUPPORT THE VALIDITY OF CLASSIFICATION OF CHLAMYDIAE BY RE ANALYSIS AND PROVIDE GROUNDWORK FOR CLASSIFYING CHLAMYDIAE BY MONOCLONAL ANTIBODY TECHNIQUES.

AN IMPORTANT ADDITIONAL FINDING OF THE FIRST STUDY WAS THE LONG TERM CHLAMYDIAL INFECTION OF TURKEY LATERAL NASAL GLANDS. SUBSEQUENT STUDIES HAVE SHOWN THAT SAMPLING OROPHARYNGEAL SWABS FOR DETECTION OF CHLAMYDIAL ANTIGEN IS MORE RELIABLE THAN THE COMMONLY USED METHOD OF SAMPLING CLOACAL SWABS OR FECES. THIS TECHNIQUE SHOULD REDUCE THE NUMBER OF FALSE NEGATIVE DIAGNOSES IN TURKEYS AND PET BIRDS.

THE INABILITY TO REISOLATE OR DETECT B577 STRAIN CHLAMYDIAE IN TISSUE SECTIONS BY IMMUNOPEROXIDASE STAINING SUGGESTS THAT MAMMALIAN CHLAMYDIAL STRAINS DO NOT INFECT BIRDS. SIMILAR FINDINGS WERE SEEN IN WILD BIRDS INOCULATED WITH THE B577 STRAIN; THESE BIRDS ALSO DID NOT SEROCONVOLVE TO CHLAMYDIAL ANTIGEN. COMPARISON OF MAMMALIAN AND AVIAN STRAIN MOMP VARIABLE DOMAIN SEQUENCES MAY REVEAL AVIAN-SPECIFIC PEPTIDES THAT WOULD BE IMPORTANT IN VACCINE DESIGN.

THE SECOND STUDY SHOWED THAT HOST CELL ENDOSOMES
containing viable chlamydiae are only slightly acidified. Heat-killed EBs were unable to cause inhibition of endosomal acidification. Because endosomes containing heat-killed (versus live) EBs were seen fusing with lysosomes, this study indicates that inhibition of endosomal acidification is the mechanism by which chlamydiae inhibit fusion of EB-endosomes with lysosomes. This is consistent with the fact that other intracellular pathogens that reside in endosomes that don't fuse with lysosomes (i.e. Toxoplasma gondii, Legionella pneumophila, and Mycobacterium tuberculosis) also inhibit endosomal acidification. 

Results of the second study also indicate that molecules on the outer membrane of the EB are responsible for effecting the block of endosomal acidification. Heat inactivation would denature molecules on the surface of the EB that are important in establishing the acidification block. One could hypothesize that a chlamydial outer membrane molecule binds to and recruits plasma membrane Na⁺,K⁺-ATPases into EB endosome membranes. The increased interior positive charge provided by these ATPases would form an electrical barrier against accumulation of protons.

Because antigen processing is dependent on endosomal acidification and fusion of endosomes with lysosomes, it is probable that processing of chlamydial antigens is inhibited by viable chlamydiae. Eventual stimulation of the immune
system may occur by the processing of, and presentation of antigen from, small numbers of defective EBs produced during early rounds of replication. This scenario would explain the inconsistent and variable immune response seen in chlamydia-infected turkeys.92

The next step in determination of the mechanism of chlamydial inhibition of endosomal acidification would be to establish that Na⁺,K⁺-ATPases are in the EB endosomal membranes. This could be done directly (if monoclonal antibodies are available) by immunoelectron microscopy or by isolation of membranes from EB endosomes (by ultracentrifugation) and Western blotting. Indirect evidence could be gained by incubation of chlamydia-infected cells with ouabain followed by DAMP labeling. Since ouabain inhibits endosomal Na⁺,K⁺-ATPases, acidification of endosomes containing live EBs would be expected. If the Na⁺,K⁺-ATPase is proved to be one of the receptors for chlamydial ligands, construction of chlamydiae deficient in these ligands may result in an effective vaccine.
<table>
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<th>Characteristic</th>
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<th>Lysosomes</th>
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<tr>
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<td>-</td>
<td>Mayorga et al., 1989(^\text{116})</td>
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<td>-^</td>
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<td>+</td>
<td>?(^\text{?})</td>
<td>?</td>
<td>Mayorga et al., 1989(^\text{117})</td>
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* = Yes  
† = No  
‡ = Unknown  
* Guanosine 5'--O-(3-thiotriphosphate)  
† N-ethylmaleimide-sensitive cytosolic factor  
‡ ras genes from rat brain
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