Characterization of humoral antibody response in turkeys experimentally exposed to *Chlamydia psittaci*

by

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GENERAL INTRODUCTION

Chlamydia psittaci comprise a diverse group of bacterial pathogens of birds, mammals, and lower vertebrates. They have been associated with pneumonitis, conjunctivitis, placental infections and abortions, genital infections, mastitis, encephalomyelitis, polyarthritis, polyserositis, and enteritis. In birds, *C. psittaci* produces a naturally occurring, systemic, contagious, and occasionally fatal disease. Currently, the disease in turkeys, ducks, pigeons, and caged birds has received increased attention because of the economic losses and the potential health hazard to humans in contact with diseased birds.

Surface components of chlamydiae that participate in early stages of infection include antigens with genus-, species-, and subspecies-specificity. Some of these induce specific antibodies during infection. These antigens of *C. psittaci* have not been studied in enough detail to allow determination of their role in turkey infection.

The purpose of this investigation was to: 1) identify outer membrane proteins (OMPs) which stimulate immunoglobulin G production in experimentally *C. psittaci* infected turkeys, and 2) compare immunochemical properties of OMPs from avian and mammalian *C. psittaci*.

The investigation consisted of an integrated immunochemical-biophysical and ultrastructural approach on

OMP characterization of C. psittaci. Representative strains of distinct C. psittaci groups as defined by biological, restriction endonuclease, and monoclonal antibody specificity analyses were examined. The strains included TT3 (virulent turkey), VS1 (psittacine), and B577 (ovine abortion) isolates. Turkeys were experimentally infected with the isolates and plasma or serum were collected at regular intervals for 142 days. The immune response was characterized by immunoblotting techniques.

Chlamydia psittaci elementary bodies were isolated through centrifugation using a renografin gradient and were mechanically disrupted by ultrasound into a highly fragmented state. Further purification was accomplished by differential centrifugation, and cell walls were disaggregated by sarkosyl (detergent). These outer membrane-enriched preparations were fractionated by ultracentrifugation into particulate and soluble fractions. The particulate fraction containing outer membranes was treated with nucleases. Outer membrane protein-enriched fractions were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using antibodies from experimentally infected turkeys.

Immunoblotting was used to characterize circulating antibodies to specific OMPs from elementary bodies of C. psittaci in turkeys. It was found that the major OMP, the 60K proteins, and a 97.4K OMP were the predominant antigens

recognized by IgG from turkeys which had been experimentally exposed to a virulent avian (turkey) strain of C. psittaci. Immunoglobulin G profiles from joint fluids were very similar to those obtained from plasma samples which were collected on the same post-inoculation day. In addition, antibody reactions with antigens from specific strains of C. psittaci using sera from turkeys experimentally exposed to avian or mammalian chlamydiae were demonstrated. This study may provide necessary information for vaccine and diagnostic studies of C. psittaci infection in turkeys, pet birds, and mammals. Also, it may be beneficial in serotyping specific C. psittaci strains.

This thesis is presented in the alternate format and consists of two manuscripts to be submitted for publication. A review of the literature precedes the first manuscript. All literature cited appears in the reference section which is located after the second manuscript. The candidate was the principal investigator and author for each manuscript presented.

LITERATURE REVIEW

Avian chlamydiosis

Avian chlamydiosis is a systemic and occasionally fatal disease of birds. It is caused by the bacterium Chlamydia psittaci which includes a number of serologically distinct strains. Over 120 species of birds are known to be susceptible to the organism. Economic and public health concerns are usually associated with the disease in turkeys, pigeons, ducks, or pet birds. In turkeys (Meleagris gallopavo), respiratory disease and septicemia frequently occur causing significant economic losses for the poultry industry, particularly when birds are housed under conditions of overcrowding and poor sanitation (119). Mortality may reach 25-30% in some flocks of turkeys (119, 7).

Avian chlamydiosis (psittacosis, ornithosis) is an acute or chronic disease of wild and domestic birds, characterized by respiratory and systemic infection, and transmissible to other birds, mammals, and humans. In turkeys, systemic chlamydial infections may lead to pneumonia and airsacculitis, pericarditis, enteritis with diarrhea, arthritis, and conjunctivitis (119). Lesions include a mixture of vascular, cellular, and degenerative changes (16). Lungs are hyperemic, edematous, and have thick, cloudy, and edematous air sacs. Serous surfaces of the air sacs, pericardium, liver, and intestine are often covered with

fibrinous exudates. Single and clustered chlamydial cells are seen in Giemsa-stained smears or sections of trachea, lungs, and air sacs as well as in other affected tissues; and intracellular forms may be found in macrophages.

Inhalation of C. psittaci-contaminated particles is believed to be the primary natural route of entry for initiating chlamydiosis in turkeys (119). Chlamydia psittaci can be cultured from respiratory discharge and digestive dejecta of infected birds and may be isolated from dust and litter in poultry confinement housing operations which previously housed infected birds. The source of infection to a new flock of turkeys is thought to be dried excrement from wild birds commingling with the flock, or infected birds held over on the farm or introduced into the flock (119).

The epidemiology of this disease is not completely understood. Since chlamydial infections in turkeys are not conveniently controlled, sporadic outbreaks continue to occur (44). Many factors (e.g., environmental stress, secondary infection) may contribute to susceptibility or severity of infection by C. psittaci in turkeys (119, 37, 8). Stress may trigger reoccurrence of the disease in carriers, which then may become a source of chlamydiae, as clinically-inapparent intestinal infections of these birds usually lead to prolonged fecal shedding (143).

Sporadic outbreaks, especially in turkeys, are believed to originate from migratory birds. Primary outbreaks in

Texas during 1974 and 1975 coincided or closely followed the south-to-north and north-to-south migrations of wild birds (63). In support of this theory Bracewell and Bevan (24) demonstrated that complement fixation titers (CFT) of serum from fully housed chickens were negative. In comparison, low, consistent CFT were found in turkeys and ducks which were partly kept in the open. The highest rates were found in geese which were not housed in buildings.

Over 120 wild avian species (representing 10 orders) have been shown to sustain and excrete highly virulent strains of chlamydiae (97). Shedding of C. psittaci in feces of experimentally-infected wild birds (126) and chickens (10) has been reported. Experimental transmission of C. psittaci to turkeys from wild birds has been demonstrated (60, 56). There is no conclusive evidence of transovarian (40, 117) or arthropod (119) transmission. However, chlamydiae have been recovered from nest mites which remained 3 months after the removal of turkeys (45); the relationship of mites to the transmission of chlamydiae to turkeys needs further investigation.

Chlamydia psittaci is an occupational health hazard in the poultry industry. It may lead to pharyngitis and pneumonia with chlamydemia and systemic involvement in humans. The first epidemic of human chlamydiosis associated with the processing of turkeys was reported in 1948, in which three deaths occurred out of twenty-two cases (78). More

recently, numerous outbreaks have been documented (44, 6). In 1986, approximately 170 packing plant employees became infected with C. psittaci during outbreaks in Minnesota*. Some of these employees were hospitalized.

Chlamydiae

Classification. Chlamydia psittaci is a gram-negative, nonmotile, coccoid (0.2 - 1.5 um), obligate intracellular bacterium which multiplies only within membrane-bounded vacuoles in the cytoplasm of host cells by means of a unique developmental cycle characterized by change of infectious, small elementary bodies (EB) into noninfectious, larger reticulate bodies (RB) that divide by fission (102, 136, 18). Functional and morphological differences between EB and RB have been well characterized.

The order Chlamydiales consists of one family (Chlamydiaceae) of a single genus, Chlamydia, with two species: psittaci and trachomatis. Acceptance of a third species, "pneumoniae" (TWAR), has been proposed (39, 54). Generally, C. trachomatis is sensitive to sulfonamides and D-cycloserine and produces iodine-staining cytoplasmic inclusions which contain glycogen. In contrast, C. psittaci is not inhibited by sulfonamides or D-cycloserine and inclusions in cytoplasmic vesicles contain no glycogen and

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*Andersen, A. A. National Animal Disease Center, Ames, Iowa: Personal communication, 1987.

are not stained by iodine. Morphology of inclusions is also helpful in the differentiation of C. trachomatis and C. psittaci (102). Inclusions of C. trachomatis are oval and often not completely filled with chlamydial cells. In contrast, C. psittaci inclusions are irregular or diffuse and usually packed with chlamydial cells.

Chlamydia trachomatis includes the organisms causing trachoma, inclusion conjunctivitis, lymphogranuloma venereum, other sexually transmitted infections, and some murine pneumonia strains (133). These organisms have been grouped into three biovars: 1) trachoma, 2) lymphogranuloma venereum (LGV), and 3) mouse pneumonitis.

Chlamydia psittaci infects a wide spectrum of vertebrate hosts which include birds and mammals. A wide variety of diseases may be produced, e.g., polyarthritis, polyserositis, placental infections and abortions, genital infections, mastitis, pneumonitis, encephalomyelitis, hepatitis, conjunctivitis, and enteritis (119, 143). Sometimes subclinical communicable disease involving humans does occur.

The TWAR strain (C. "pneumoniae") of chlamydiae, which has been proposed as a new species, is transmitted person-to-person without involvement of an avian or animal source (130). In Finland, Nova Scotia, and Seattle, Washington (USA), serological evidence of TWAR infection was found in college students with acute pneumonia (130, 55) and the organism was isolated from students who had no previous avian

exposure (55).

Chlamydiae have a mole % guanine + cytosine (G+C) of 41-44 and its genome (double stranded) is 4-6 x 10^8 daltons (102). Both EBs and RBs contain DNA and RNA, but the RNA-to-DNA ratio is higher in RBs than in EBs (89, 147). This difference in ratios reflects the relative deficiency of ribosomes in EBs and the greatly increased protein biosynthetic activity of RBs. Deoxyribonucleic acid hybridization studies have shown that DNAs of C. psittaci and C. trachomatis hybridize only to the extent of about 10% (39, 83). Strains of C. trachomatis biovar LGV and biovar trachoma have nearly 100% homologous DNAs (39, 83, 159), but a single strain of the mouse biovar is only 30 to 60% homologous with the other two biovars. Chlamydia "pneumonia" (TWAR) have less than 10% DNA homology with either C. trachomatis or C. psittaci strains (39, 54).

Growth of organism. The growth cycle of chlamydia is initiated by EBs which survive extracellularly as metabolically dormant, spore-like (develop under favorable conditions) particles with associated infectious properties (18). Elementary bodies enter (attach, penetrate, and infect) the cytoplasm of a susceptible host cell and then develop into vegetative structures called RBs. The RBs replicate, pass through an intermediate stage (IB), and develop into EBs (EB progeny). The chlamydiae are released

from host cells and EBs reinitiate the cycle of infection.

Chlamydiae lack the ability to synthesize the high-energy compounds adenine triphosphate (ATP) and guanine triphosphate (GTP) (158, 160). Thus, they parasitize their host for ATP. Chlamydiae catabolize glucose-6-phosphate (but not glucose) to pyruvate and pentose to a limited extent but without producing useful energy by the Embden-Meyerhoff and pentose phosphate pathways, respectively (158, 160). Adenine triphosphate is formed in the host cell by the oxidation of fuel molecules (oxidative phosphorylation) which takes place in the inner membrane of mitochondria (often found in close proximity of chlamydial inclusions). It has been suggested that chlamydiae has an ATP-ADP translocation mechanism which is in the reverse manner of mitochondria (67). The decreased ATP level of a chlamydia-infected cell inhibits lysosomal protein degradation, since much energy is needed for the protein pump to maintain acidic conditions necessary for activity of hydrolytic enzymes and to ensure lysosomal integrity (157).

Studies have shown that one mechanism by which the host inhibits the growth of chlamydiae may be the induction of tryptophan deficiency in infected cells (31, 65, 66, 80). Gamma interferon is produced in response to chlamydial infection and also induces tryptophan dioxygenase activity in human fibroblasts. Chlamydiae are unable to replicate in interferon-treated cells, but addition of high concentrations

of tryptophan to the incubation medium reverses the effect of interferon. These studies indicate a requirement for cellular factors that are influenced by interferon.

Diagnosis. Tissues or body fluids containing chlamydiae are used for isolation. The organism may be propagated in yolk sac of chicken embryos, in mice by nasal instillation, in guinea pigs by intraperitoneal inoculation, and in tissue cell culture. The adaptation of chlamydiae to grow in susceptible tissue culture cells in defined media has made it possible to study the development and chemical characterization of C. psittaci and C. trachomatis.

Demonstration of chlamydial inclusions in tissue cell culture is the standard for establishing laboratory diagnosis of chlamydiae. Infectivity is enhanced by centrifugation of the inoculum onto tissue culture monolayers. Antibiotics (e.g., streptomycin, vancomycin, gentamicin, mycostatin) are commonly used to suppress growth of contaminating bacteria from heavily contaminated specimens. Broad spectrum antibiotics such as tetracycline, penicillin, or macrolides must be excluded since they will inhibit the growth of chlamydiae. Cyclohexamide, a glutarimide antibiotic which inhibits the peptidyl transferase activity of the 60 S ribosomal subunit (thus preventing protein synthesis in eucaryotic cells but not prokaryotic cells), is also routinely added to the medium (1, 144). Parallel cultures

are kept: one is used for subculture and the other is stained for visualization of inclusions in cytoplasm.

Chlamydial plasmids have been isolated and cloned into Escherichia coli. In C. trachomatis all serotypes carry a common plasmid; therefore, a useful diagnostic probe has been developed for the detection of this plasmid (77). The application of in situ DNA hybridization in the diagnosis of human chlamydial infection in biopsied tissue sections has also been described (74). However, plasmids are not found in all C. psittaci strains. A probe generated from a psittacine (cockatiel) C. psittaci plasmid has been used for hybridization studies (94). Homologous DNA sequences were detected in parrot, pigeon, and duck strains of C. psittaci, but not in ovine abortion strains.

Bacteriophages have potential in the development of genetic probes. An avian (duck) chlamydial bacteriophage has been described by Richmond et al. (125). These viruses were isolated through cesium chloride gradients from infected monolayers and successfully transferred from one chlamydial strain to an another (21). This work is significant in that it may provide an effective approach for transfection of chlamydiae.

Restriction fragment length polymorphisms in genetic material (DNA) cut with restriction endonucleases have provided definitive "finger-printing" of chlamydial strains. Andersen has proposed that several isolates of C. psittaci be

placed into virulent-turkey, psittacine-like, and mammalian groups on the basis of restriction endonuclease analysis (REA) and serology using monoclonal antibodies (3, 5). McClenaghan et al. (93, 94) have also demonstrated by REA four distinct electropherotypes with ovine abortion and arthritis strains and avian psittacine and columbiforme strains of C. psittaci. In addition to REA, these groups could also be separated on the basis of biological activity in turkeys (149) and reaction to strain-specific monoclonal antibodies using indirect fluorescent antibody staining techniques (5).

Previous efforts in serotyping isolates of C. psittaci have reflected the diversity of this organism. Schachter et al. (134, 135) classified ovine and bovine isolates into two serological types by using plaque reduction neutralization assays. Perez-Martinez and Storz (122) distinguished nine immunotypes by immunofluorescent staining techniques with conventional antisera. Others (51, 139) have grouped C. psittaci into serovars by using a panel of monoclonal antibodies against specific antigens.

Genus-, species-, and strain-specific antigens of chlamydiae have been detected by indirect- and micro-complement fixation (116, 124), indirect fluorescent antibody staining (155), plaque reduction neutralization (134, 135), latex agglutination (59), agar gel precipitin (115), and enzyme-linked immunosorbant (82, 85, 129) assays. Typing

procedures based on strain-specific antigens of C. trachomatis include enzyme-linked immunosorbant assay (12), radioimmunoassay (13, 110, 141), and microimmunofluorescence (141, 156). Grouping by microimmunofluorescence (155) has allowed the classification of C. trachomatis into fifteen serovars (152). Monoclonal antibodies have also been developed for immunotyping C. trachomatis using microimmunofluorescent staining (156). These serovars were placed into one of three biovars: 1) trachoma, 2) LGV, and 3) mouse.

Chlamydiae possess a common genus-specific antigen (group or complement fixing antigen) which can be extracted by ether (42) or mild ionic detergents such as sodium dodecyl sulfate (20) and sodium deoxycholate (81). Antigenic activity of the sodium deoxycholate extract is not diminished by heat (100° C for 30 minutes) and pronase. Sodium meta-periodate, which oxidizes reactive epitopes located in the carbohydrate region of the lipopolysaccharide molecule, does interfere with the antigenic activity. These properties indicate the lipocarbohydrate nature of the immunodominant antigen of this genus-specific chlamydial antigen complex. Sodium deoxycholate extract of concentrated suspension of chlamydia propagated in chicken embryo is routinely used in complement fixation and agar gel immunodiffusion assays (115).

Serological methods routinely used for detecting antibodies against chlamydiae in turkey serum include

complement fixation, agar gel immunodiffusion, and the latex agglutination assays (9, 57, 58, 59, 87, 115, 116). The most widely used method for diagnosing chlamydial infection in turkeys is the complement fixation test. Demonstration of a fourfold or greater increase in titer of paired sera is considered diagnostic of a current infection. Serology of diseased flocks from both high- or low-virulent strain outbreaks show greater than 90% of the birds with antibodies against the chlamydial group antigen complex by the time the clinical disease appears in the flock (119). The correlation of complement fixing antibody titer to immunity has not been demonstrated even though antibody is important in neutralization (32, 35, 121) and opsonization (164, 165) of chlamydia in vitro. Recently, enzyme-linked immunosorbant assay (ELISA) methods have been developed to demonstrate chlamydial antibodies in avian serum (129, 137). However, standards for interpreting the results have not been established.

Chlamydial virulence

Specific strains of C. psittaci are associated with distinct pathological syndromes of chlamydiosis in turkeys (16, 17, 112, 113, 114, 117, 118, 119, 149). Highly virulent strains cause acute epornitics in which 5 to 30% of affected birds die. These strains produce necrotizing lesions characterized by extensive vascular congestion and

inflammation of vital organs. Virulent strains may also be transmitted to humans associated with the processing of these turkeys. In contrast, low-virulent strains produce progressive epornitics with less than 5% mortality when uncomplicated by secondary bacterial or parasitic infection. This disease is characterized by chlamydial proliferation resulting in formation of fibrinous exudate or plaques and usually does not develop into the severe vascular damage evident in birds infected with virulent strains. Humans are rarely infected with low-virulent C. psittaci unless unusual conditions alter the balance between infection and resistance (119).

Titration of high-virulent turkey strains in experimental animals and birds indicate that these strains are highly infectious and lethal for mice, guinea pigs, turkeys, and parakeets but innocuous to pigeons and sparrows (119). In contrast, low-virulent strains have high infectivity but low lethality for mice, pigeons, sparrows, and turkeys. Guinea pigs are usually not affected. Recently, an 8-day-old chicken model of systemic and acute infection has demonstrated avian C. psittaci to be more pathogenic than mammalian C. psittaci (145).

Low-virulent strains of C. psittaci are often isolated from pigeons, ducks, and occasionally turkeys, sparrows, and other wild birds. Pigeons are susceptible to a variety of these low-virulent strains from pigeons and sparrows, and to

some mammalian strains. However, pigeons are innocuous (excrement shedding occurs) to virulent turkey strains and to some strains from domestic herbivores (119). Therefore, pigeons and other birds not only serve as a potential reservoir of virulent turkey strains but seems to be protected by a mechanism which prevents certain strains from damaging their tissues.

Chlamydia psittaci strains TT3 (virulent turkey), VS1 (parrot), and B577 (ovine abortion) are representative isolates from virulent turkey, psittacine-like, and mammalian C. psittaci groups, respectively, as defined by Andersen's restriction endonuclease analysis (3) and monoclonal antibody specificity studies (5). In addition, they exhibit differences in terms of natural and experimental pathogenicity for wild, domestic, and laboratory animals (16, 17, 112, 113, 114, 117, 118, 119, 149). Differences among these strains in virulence for turkeys have been determined by mortality rate and/or lesion scores (149, 119, 114). Tappe et al. (149) have compared gross, light, ultrastructural, and clinicopathological changes induced in turkeys by these representative isolates. Birds infected with TT3 were lethargic; and had decreased body weight, airsacculitis, bronchopneumonia, and severe pericarditis. Turkeys infected with VS1 had severe airsacculitis and bronchopneumonia, but a low incidence of pericarditis. The only sign detected in B577-inoculated birds was mild

peribronchial pneumonia.

Chlamydial outer membrane

Surface components of EBs are involved in cellular interactions (e.g., attachment, internalization, inhibition of phagosome-lysosome fusion, synthesis of chlamydial macromolecules by "energy-parasitic" mechanisms) between host and parasite (101, 120, 136). Outer membrane proteins (OMPs), as well as many other virulence determinants of bacteria (29, 163), are cell surface components or extracellular products which include capsules, toxins, enzymes, flagella, pili, and siderophores. Outer membrane proteins are associated with bacterial virulence since they are ultimately involved in the initial interaction between host and parasite. Synthesis or secretion of virulence determinants can be affected by growth conditions or growth stage (28, 106).

There has been considerable interest and progress in the study of the contribution of OMPs to virulence. The virulence of gram-negative bacterial pathogens is often associated with the expression of certain OMPs (28, 29, 163). Comparisons between strains having specific OMPs with those that do not provide evidence that surface components are involved in the pathogenesis of disease. Experiments with isolated, purified OMPs confirm immunological activity of either the original organism from which the component has

been removed or an isogenic strain lacking the gene(s) that encodes for that component (recombinant strain). Inhibition of host response by treatment of test organisms with antibody to that component or performing biological assays in its presence also confirms reactivity.

Outer cell wall complexes similar to those of free-living gram-negative bacteria have been described for chlamydiae (34, 49, 70, 72, 148). Both gram-negative bacteria and chlamydiae have a cytoplasmic and an outer membrane (148) which is disrupted by ethylenediamine-tetraacetic acid (105) and by polymyxin B (also known to inhibit synthesis of peptidoglycan constituent of bacterial cell wall) (91). No muramic acid (or any other amino sugar which might have replaced it in the peptidoglycan subunit) has been found (53, 11). There is no detectable peptidoglycan; instead outer membranes of EBs are disulfide cross-linked, while those of reticulate bodies (RBs) are not (62, 68, 69, 106, 109). Both EB and RB cell membranes are sensitive to penicillin (92), which blocks cell-wall synthesis (48, 52). In addition, growth and multiplication of chlamydiae are inhibited by bacitracin (75) and D-cycloserine (104, 86). Since chlamydial replication is sensitive to these antibiotics, it has been suggested that chlamydial envelopes contain D-alanyl-D-alanine peptides that are crosslinked to structures other than peptidoglycan (53).

The initial stage of chlamydial internalization is attachment of bacteria to mucosal surfaces or individual target cells. The organism may be expelled by mucociliary escalation or phagocytized by macrophages or nonprofessional phagocytes. The mechanisms involved in translocation of chlamydia from the outside to the inside of a host cell is not known. After entry into the host cell, chlamydia inhibits phagosome-lysosome fusion. Evasion of lysosomes is observed for ingested chlamydia, but not for heat- or antibody-treated chlamydia (49). Likewise, isolated EB cell walls do not provoke phagosome-lysosome fusion in macrophages, but if the cell walls are heated before ingestion, they no longer inhibit fusion (46). Nevertheless, chlamydiae must be internalized by the cell and then be able to compete with the host for intracellular nutrients and precursors necessary for development and survival.

The accuracy of chemical and molecular analysis of OMPs depends on the availability of highly purified, homogenous preparations. Chlamydiae are separated from cellular debris through gradients [e.g., sucrose (146, 100), renografin (76), percoll (108)]. Various detergents have been used to extract proteins from cells. For example, sarkosyl, an ionic detergent, selectively solubilizes the cytoplasmic (inner) membrane of Escherichia coli under conditions in which Triton X-100 and sodium dodecyl sulfate solubilize most cell membrane (inner and outer) proteins (47).

Chlamydial OMPs can be purified because of their insolubility in sarkosyl (34). The sarkosyl-insoluble fraction of chlamydia contains a major OMP (MOMP) of approximately 40 kilodaltons (K) which constitutes approximately 60% of the total protein of the OMP-enriched fraction (34, 131). All strains possess a MOMP which varies between strains by approximately four kilodaltons in molecular mass from about 39 to 43K (108, 50).

Synthesis of several OMPs of EBs is regulated by processes which coincide in time with the transformation of RBs into EBs, and this may be very important in the development of infectious EB progeny (106, 61, 161, 68, 69, 132). The outer membrane of EBs contain a 60-62K, 59K, and a 12 to 12.5K protein which are easily labeled with [^{35}S] cysteine. These proteins have been termed "cysteine-rich" proteins since cysteine is incorporated during EB development (68). These studies demonstrated the involvement of cysteine-containing polypeptides in structural differences between EBs and RBs which may be involved in EB rigidity.

DNA-binding proteins of chlamydiae have been proposed to be involved in mediating fundamental changes in nucleoid reorganization of the chlamydial nucleoid (154). The 17K EB-specific nucleoprotein is a prominent, unique DNA-binding protein band of EB chromosomes but not RB chromosomes. Therefore, a highly condensed EB chromosome is largely inaccessible to chlamydial transcriptional and translational

processes, thus constituting the metabolically-dormant stage of development.

Adhesion has been defined as a relatively stable, essentially irreversible attachment of bacteria to surfaces and the term "adhesin" denotes the structure which mediates adhesion (79). In C. trachomatis, adhesin proteins of 15 to 18 and 29 to 32 kilodaltons (K) in molecular mass have been described (15, 61, 161). A protein of 18K has been suggested to mediate adherence of chlamydiae to tissue culture cell surfaces as the initial step for internalization of chlamydial pathogenesis (15).

A 57K chlamydial protein common to all fifteen serovars of C. trachomatis and to C. psittaci strains GPIC and Mn shares antigenic determinants with similar molecular weight proteins (often called "common antigen") which have been described in many other gram-negative bacteria (96, 99). In a guinea pig model of inclusion conjunctivitis, the 57K protein was a potent stimulator of ocular delayed hypersensitivity when placed topically onto the conjunctiva of ocular immune guinea pigs (99). However, it does not seem to be the 60K cysteine-rich protein which is found in the sarkosyl-insoluble outer membrane-enriched fraction and is a dominant immunogen in chlamydial infections of guinea pigs (14), monkeys (36), and humans (108).

Recently a number of studies have compared protein profiles of invasive and non-invasive strains of C. psittaci.

Consistent differences between ovine groups have been reported, the most pronounced being a 90K band from invasive strains (128). Winsor and Grimes (162) have demonstrated that variations in molecular weight of the MOMPs of avian C. psittaci correlated with differences in infectivity and cytopathology of L-929 tissue culture cells. In addition, rabbit hyper-immune sera, which were produced against some of these isolates, reacted with the 97K and 60K antigens of both high- and low-infectivity groups, but reacted only with the MOMP of isolates from their respective groups.

Outer membranes of chlamydiae contain lipopolysaccharide (LPS) and phospholipids (33). Chlamydial LPS contains an epitope that is unique to both C. psittaci and C. trachomatis (33), but also contains at least two determinants which cross-react with many gram-negative bacteria (111). Lipopolysaccharide appears to possess an additional epitope which is more closely associated with the MOMP (34, 70, 131) in EBs relative to RBs (22). The major phospholipid found in EBs is phosphatidylethanolamine with phosphatidylcholine as the next abundant (107). Significant amounts of diphosphatidylglycerol and phosphatidylglycerol have been found along with other phospholipids present in small amounts which includes phosphatidylinositol, sphingomyelin, and phosphatidylserine. No lysophosphatidylcholine (destabilizes lipid bilayers) have been detected.

Brade et al. (25) described some differences between the LPSs extracted from an ovine abortion strain of C. psittaci and a strain of C. trachomatis. Their analysis showed additional hexosamine and D-galactosamine in the LPS of C. psittaci which were not found in the LPS of C. trachomatis. In addition, a slower migration rate of C. psittaci LPS than C. trachomatis LPS was shown by silver-stained sodium dodecyl sulfate-polyacrylamide gel. Furthermore, they proposed that C. psittaci possesses both a genus-specific epitope and a species-specific determinant located in the carbohydrate moiety of the LPS molecule.

Immunoblotting

Immunoblotting (151) permits precise identification of the antigen(s) recognized by an antibody or antiserum. The location of various epitopes on specific macromolecules can be determined from the analysis of sera from diseased or immunized animals (also humans) and monoclonal antibodies by using immunoblotting techniques. Proteins are separated on the basis of molecular weight using polyacrylamide gel electrophoresis. Reducing agents, e.g., 2-mercaptoethanol which prevents aggregation via interchain disulfide bonds, have been commonly used in separating proteins through sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins are then transferred (or blotted) from the gel matrix to a membrane surface (e.g., nitrocellulose) by passive diffusion,

covalent immobilization, or electric current. On the membrane surface, proteins are easily accessible to a broad variety of probes.

The use of high quality probes is essential. This will strongly influence the specificity, validity, and significance of the final result. These probes must be rigorously tested using the traditional types of controls for sensitivity and specificity. Labeling allows for easy identification of the probed structure. Results are determined by the ratio of the specific staining to the low background staining.

Immunoglobulins (i.e., IgG) have successfully been applied for specific demonstration of antigens due to their high affinity and specificity existing between antibodies and antigens. Protein A (from Staphylococcus aureus), which interacts with the Fc region of IgG from most mammals, has also been useful in immunocytochemistry for the localization of antigenic sites by indirect staining techniques (19, 41, 127).

Studies using monoclonal antibodies against C. trachomatis surface antigens indicate genus-, species-, and subspecies-specific antigens are predominately associated with the MOMP (12, 13, 90, 141). All strains of Chlamydia contain the identity genus-specific antigen (complement fixing antigen), but differ in species and strain antigen specificity due to their cell wall antigens. Immunoblotting

has shown that the MOMP (approximately 40K) reacts with the serovar-, subspecies-, and species-specific monoclonal antibodies; and genus-specific monoclonal antibodies react with the LPS or with no bands.

Immunoblot experiments by Newhall et al. (108) have shown that antibodies to the 60K and 62K proteins were dominant in the majority of sera from individuals with genital tract infections of C. trachomatis. McClenaghan et al. (94) have demonstrated that three relatively high molecular weight polypeptides (78-90K) gave the most marked antibody response with sera from sheep vaccinated with an ovine abortion strain of C. psittaci. Antibody response to specific chlamydial antigens with regard to trachoma infection has been detected by immunoblot analysis on sera and tears from monkeys infected with a human strain of C. trachomatis (36). Also, in tears from monkeys with primary acute C. trachomatis serovar B conjunctivitis, IgA response to MOMP was predominantly to subspecies-specific B complex.

The guinea pig has been used as a model for human genital infections (14). Immunoglobulin G and IgA from ocular secretions were detected by immunoblot analysis shortly following a primary infection in the genital tract (ocular infection absent). Serum IgG to the MOMP appeared approximately 12 days post-infection, peaked at 20-30 days, declined between 50 and 90 days, and remained relatively unchanged as long as 825 days. Antibodies to the 61K protein

developed approximately the same time as the MOMP but continued to increase while the anti-MOMP antibodies decreased. Immunoglobulin A from genital secretions was also detected approximately 20 days post-infection for both the 61K and MOMP, but was minimal by the thirtieth day. In addition, antibody responses to other membrane components and their various patterns of waxing and waning were noted during the observation period.

Chlamydial surface proteins which are immunogenic have not been studied in detail to allow precise determination of their role in turkeys infected with C. psittaci. Immunoblot techniques which detect chlamydial antigens have demonstrated the presence of circulating antibodies to chlamydiae OMPs from humans (108), sheep (94), monkeys (36), and guinea pigs (14) infected with chlamydiae. Chlamydial proteins may provide excellent immunogens for vaccination and/or antigens for in vitro immunological studies.

PART I. CHARACTERIZATION OF CIRCULATING ANTIBODIES AGAINST *CHLAMYDIA PSITTACI* IN TURKEYS

ABSTRACT

Sequential plasma and joint samples from turkeys experimentally inoculated with Chlamydia psittaci TT3 were evaluated by immunoblotting to identify chlamydial antigens eliciting antibodies (ABs) during the course of infection. The objective was to identify chlamydial antigens stimulating host immune response during infection, as some of these antigens may induce production of protective ABs. Protein antigens from elementary bodies of TT3 were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred electrophoretically to nitrocellulose before being probed with plasma or synovial joint fluid from TT3-inoculated birds. The major outer membrane protein (MOMP), the 60 kilodalton (K) proteins, and a 97.4K outer membrane protein (OMP) were the predominant antigens recognized by IgG in the plasma and joint fluids. Plasma IgG specific for the 97.4K protein band was first detectable at post-inoculation day (PID) 10 while other OMPs were usually detected later. Antibodies to the 60K and MOMP were variable and were first detected as early as PID 14-17 and PID 7-10 in some birds and as late as PID 36-42 and PID 42-70 in others, respectively. The ABs were detectable for at least 142 days post-infection. Immunoblotting techniques showed that the antigens to which these ABs were reacting were protein in nature. These observations may have implications for the development of

serodiagnostic assays as well as the identification of potential OMPs for subunit immunogens in birds.

INTRODUCTION

Avian chlamydiosis (ornithosis, psittacosis) is an acute or chronic disease of domestic and wild birds, characterized by respiratory and systemic infection. Specific chlamydial strains are usually associated with one type of bird; however, they will infect other birds and are transmissible to humans.

Antigenic determinants associated with the chlamydial outer membrane play a significant role in the immune response since they are involved in the initial interactions between host and parasite (101, 120, 136). Surface components of chlamydiae that participate in early stages of infection include antigens with genus-, species-, and subspecies-specificity. Some of these induce specific antibodies during infection.

Immunoblotting techniques have demonstrated the presence of circulating antibodies to chlamydiae membrane proteins in humans with genital infections of Chlamydia trachomatis (108), in sheep vaccinated with an ovine abortion strain (94), in monkeys experimentally inoculated with a human trachoma strain (36), and in guinea pigs which were used as a model for human genital infection using the guinea pig inclusion conjunctivitis (GPIC) strain of C. psittaci (14). Antibodies to antigenic determinants of C. psittaci have not been examined in detail to allow precise determination of

their role in turkey infections.

In the present study, we used an immunoglobulin G (IgG) immunoblotting assay to examine turkey humoral antibody response against outer membrane proteins (OMPs) from elementary bodies (EBs) of C. psittaci during the course of experimental infection with the virulent avian (turkey) strain TT3.

MATERIALS AND METHODS

Turkeys

Nonvaccinated, ten-week-old broad-breasted white turkeys were used. Group 1 birds were inoculated intramuscularly with 10^4 IFU of C. psittaci TT3 (fifteen birds). Group 2 were not inoculated and used as pen-contact birds (five birds). Both groups were housed on litter in one room. Antibiotic-free food and water were provided ad libitum. Birds were observed daily for clinical signs. Tracheal and cloacal swabs and plasma samples were collected once before inoculation, and following inoculation on alternate days for two weeks and then weekly for 18 additional weeks. Joint fluid was collected at the time of last bleeding.

Chlamydiae and cultivation

Chlamydia psittaci TT3 (Texas turkey strain; 119), VS1 (parrot strain; 5), and B577 (ovine abortion strain; 142) were obtained from the stock collection of the National Animal Disease Center, Ames, Iowa. Chlamydiae were grown in Vero cells (ATCC, Rockville, MD) in the presence of 0.5 ug/ml cyclohexamide (Sigma, St. Louis, MO). Vero cells were maintained in F15 Eagle's Minimal Essential Medium (pH 7.2) containing Earl's Balanced Salts (Gibco, Grand Island, NY), 100 mM glucose, 5% fetal bovine serum (inactivated by heating at 56° C for 30 min), 10 mM N-2-hydroxyethylpiperazine-N'-2-

ethanesulfonic acid (HEPES; Calbiochem-Behring, La Jolla, CA), 2 mM L-glutamine (Gibco), 20 ug/ml gentamicin sulfate (Scherring Corp., Kenilworth, NJ), and 4 ug/ml amphotericin B (E. R. Squibb & Sons, Inc., Princeton, NJ). Cultures were examined at 24 h intervals to detect cytopathic effects and cell necrosis (sloughing). Chlamydia were harvested when greater than 20% of tissue culture cells had died or showed cytopathic effects. Vero cells were removed by centrifugation (500 x g for 10 min). The supernatant was collected and then centrifuged (10,000 x g for 1 h). The pellet containing chlamydiae was suspended in Bovarnick's buffer (23) and then stored at -80° C until needed.

Isolation of chlamydia

Tracheal and cloacal swabs were transported in 2 ml tissue culture medium containing 500 ug/ml each of streptomycin (Pfizer Inc., NY, NY), vancomycin (Eli Lily Industries, Indianapolis, IN), and kanamycin (Bristol Labs., Syracuse, NY) and processed within 3 h after collection. Samples were vortexed and then centrifuged at 650 x g for 10 min. The supernatant was used to inoculate 96-well microtiter plates (0.2 ml/well) which contained confluent monolayers of Vero cells. Plates were centrifuged (900 x g for 1 h at 30° C), supernatant removed, and 0.2 ml tissue culture medium containing the additional antibiotics and cycloheximide (0.5 ug/ml) was added to each well. Inoculated

microtiter plates were incubated at 37° C in 3.5% CO_2. Inclusion bodies of chlamydia were detected by indirect fluorescent antibody staining (5). Vero cells were fixed with acetone:methanol (1:1) at days 3 and 6 after inoculation and incubated with a group-reactive monoclonal antibody (B577/D1; 5). Immunocomplexes were detected by using fluorescein-conjugated antimouse IgG (Cooper Biomedical, Inc., Malvern, PA). Preparations were examined under an epifluorescence microscope.

Purification of elementary bodies

Elementary bodies were partially purified by differential centrifugation followed by density gradient centrifugation in Renografin (34). Vero cells were removed by centrifugation at 500 x g for 10 min. The supernatant was collected and centrifuged at 10,000 x g for 1 h. The pellet containing chlamydiae was suspended in H_2O and centrifuged (50,000 x g for 20 min) through 35% Renografin-76 (R76; E. R. Squibb & Sons, Inc., Princeton, NJ). The supernatant was removed and the pellet was resuspended in H_2O and layered on a 35-44-51% R76 step gradient. The gradient was centrifuged (69,000 x g for 1.5 h at 4° C). The band containing EBs was collected, washed by resuspending in H_2O and pelleting (50,000 x for 20 min), and then suspended in 20 mM Tris, pH 7.6.

Preparation of outer membrane proteins

Elementary bodies were disrupted by sonication (Heat systems-Ultrasonics, Inc., Farmingdale, NY) and whole cells were then removed by centrifugation (4,000 x g for 15 min). Two percent sodium N-lauroyl sarcosinate (Sigma) in 20 mM Tris, pH 7.6, was added to the broken cell suspension (1:1), incubated 30 min at 37° C, and sarkosyl-insoluble fraction was pelleted by centrifugation (105,000 x g for 1 h at 5° C). The pellet was suspended in 10 mM HEPES, pH 7.2, containing 150 mM NaCl, 10 mM $MgCl_2$, 100 ug of RNase I-A per ml (Sigma), and 50 ug of DNase I (Sigma) per ml (22). This suspension was incubated 30 min at 37° C, centrifuged (105,000 x g for 1 h at 5° C), and the pellet (containing OMPs) suspended in H_2O. Protein concentration was determined by Coomassie brilliant blue (26) using bovine serum albumin as a standard.

Electron microscopy

Purity of EB and OMP-enriched preparations were determined by thin sectioning and negative staining techniques (27, 71). Elementary bodies were fixed with 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer (ph 7.4) for 3 h, stained in 1% osmium tetroxide for 2 h, dehydrated in graded ethanols and propylene oxide, and embedded in Medcast (Ted Pella, Inc., Tustin, CA). Sections (90 nm) were stained with lead citrate (123) and uranyl acetate (140). Ruthenium red staining was done as described above, except ruthenium

red was added (50 mg/ml) to the glutaraldehyde fixative (71).

Chlamydiae were stained with neutralized phosphotungstic acid and applied to coated copper grids with a nebulizer (27). All preparations were examined with a Philips 410 electron microscope (Philips Electronic Institute Inc., Mahwah, NJ) at an accelerating voltage of 60 kV.

Electrophoresis

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the two buffer system of Laemmli (84). Outer membrane protein-enriched preparations were heated (100° C) for 5 min in treatment buffer (2% sodium lauryl sulfate, 10% glycerol, 5% 2-mercaptoethanol, 0.003% bromphenol blue in 62.5 mM Tris, pH 6.8) before electrophoresis. Fifteen to 20 ug of protein were applied to each lane of vertical slab gels (LKB-Produkter AB, Bromma, Sweden) or 150 ug of protein was applied to the top of a preparatory slab gel. Gels were run at a constant current of 35 mA for 1.5 h and at 100 mA for 2-3 h. Protein bands were visualized by staining with silver stain (Bio-Rad, Richmond, CA) or Coomassie brilliant blue R250 (Bio-Rad). Lipid or carbohydrate bands were visualized by a silver stain which was previously shown to detect lipopolysaccharide (LPS) (73, 153). The mw for each band was obtained from a plot of mobility versus log 10 of the mw of protein standards (Bio-Rad).

Immunoblotting

Detection of antibodies against antigens of TT3 OMPs was done according to the method of Towbin et al. (151) with modifications. Chlamydia psittaci TT3 proteins were separated by SDS-PAGE (84) and electrophoretically transferred from the gel to nitrocellulose (0.45-um pore size; Schleicher & Schuell, Inc., Kene, NH). The transfer was done using a Bio-Rad Transblot cell (Bio-Rad) at 0.9 A for 20 min at 5° C in buffer containing 25 mM Tris, 192 mM glycine, 0.1% sodium lauryl sulfate, and 20% (vol/vol) methanol. Membranes were immersed in 10 mM NaH_2PO_4-0.9% NaCl, pH 7.2 (PBS) containing 0.05% Tween 20 (TPBS). The blots were either cut into strips (4mm) or placed in a Miniblotter 45 (Immunetics, Cambridge, MA) and incubated 1 h with turkey plasma or joint fluid samples (1:100). Immunocomplexes were detected with biotinylated rabbit anti-turkey IgG (H+L) (Zymed Laboratories, San Francisco, CA) followed by avidin conjugated to alkaline phosphatase with 4-chloro-1-naphthol (Bio-Rad) as the substrate. Protein specificity and efficiency of transfers were determined by associating immunoblots with individual nitrocellulose strips stained with Pelikan Fount India Ink (Pelikan AG, Hanover, Federal Republic of Germany) (64).

Major OMP (MOMP) or LPS genus-specific monoclonal antibodies (Chemicon International, Los Angeles, CA) were used as probes to identify the MOMP and the distribution of

LPS molecules among the OMPs on the nitrocellulose.

Immunological cross-reactivity of OMPs

Sera from turkeys exposed to heterologous gram-negative bacteria were used in immunoblotting. Blots of TT3 OMPs were reacted with sera from: gnotobiotic turkeys inoculated with Escherichia coli O78:K80:H9 or O2:K-:H6 (43); a turkey inoculated with Bordetella avium (supplied by Lawrence Arp, Iowa State University, Ames, IA); turkeys inoculated with Mycoplasma meleagridis, M. gallisepticum, or M. synoviae (supplied by Donna Cummins, National Veterinary Services Laboratory, Ames, IA); and a turkey vaccinated against Pasteurella multocida type 3 (supplied by Richard Rimler, National Animal Disease Center, Ames, IA). Gnotobiotic turkey sera (43) were used as negative controls.

Enzyme and periodate treatments of OMPs

Blotted nitrocellulose strips were incubated with 4 ml of trypsin (0.5 mg/ml in PBS) for 30 min at 37° C (138). The strips were washed three times with 10 ml TPBS (10 min incubation for each wash) before immunoblotting. Outer membrane proteins-enriched preparation was treated with 10 ug of proteinase K (Sigma) for 60 min at 60° C (73) or with 10 mM sodium meta-periodate (Sigma) for 18 to 20 h at 4° C (88) prior to solubilization in the SDS-PAGE sample treatment buffer and electrophoresis. Bovine serum albumin was used as

a control for the proteinase digestion and an Escherichia coli LPS (Sigma) as a control for periodate oxidation.

RESULTS

Turkeys

Chlamydia psittaci was not isolated from tracheal and cloacal swabs prior to inoculation. Chlamydiae were isolated by post-inoculation day (PID) 3-7 from both tracheal and cloacal samples from all birds inoculated with TT3 (group 1). Contact exposed birds (group 2) first showed positive isolation between PID 11-15. No isolation of TT3 was made after PID 28 from either tracheal or cloacal swabs from either group. One-contact exposed bird died PID 19 and chlamydia was cultured from this bird. Also, a TT3-inoculated bird (group 1) died PID 50.

Characterization of OMPs

The EB preparation contained less than 5% reticulate bodies as determined by electron microscopy. No bacteriophages were demonstrated by thin sectioning. Ruthenium red-stained membrane vesicles observed in the OMP-enriched preparation of TT3 is shown in Fig. 1. The diameter was determined to be 0.2-0.4 um for these vesicles and approximately 8.0 nm for the membrane diameter.

The protein profile of the sarkosyl-insoluble, OMP-enriched fraction of EBs from TT3 after silver staining for protein is shown in Fig. 2. Coomassie blue-stained gels showed similar results, but bands did not stain as intense.

Major features include a cluster of bands with relative molecular weights in excess of 85,000 (85K), a broad, diffusely staining band of approximately 60K, a 41K (MOMP), a 39K, and a 38K. The diffuse 60K band could be resolved into two bands (61.5K and 58K) by decreasing staining intensity. Few bands stained when 61.5K and 58K bands were distinguishable. Protein bands were efficiently transferred to the nitrocellulose as indicated by India ink staining (Fig. 3.A).

Immunoblot analysis of plasma antibodies

Plasma from nine of the fifteen TT3-inoculated turkeys (group 1) and from all five contact-exposed birds (group 2) were evaluated by immunoblotting. Representative immunoblots showing plasma IgG from TT3-inoculated turkeys (group 1) reacting with OMPs of TT3 are depicted in Fig. 3. The pre-inoculation plasma samples from a few turkeys gave weak reactions with the MOMP. No reactions were detected with other TT3 OMPs. Reactions detected by immunoblotting using plasma from individual birds were variable in intensity for the MOMP and 60K but were uniformly intense for the 97.4K band. Identification of immunoreactive bands was made by comparing India ink-stained OMP transblots of TT3 with immunoblots. Plasma IgG specific for the 97.4K protein band was first detectable on PID 10-14 in all TT3-inoculated birds (group 1). Antibodies to the 60K and MOMP were variable and

were first detected as early as PID 14-17 and PID 7-10 in some birds and as late as PID 36-42 and PID 42-70 in others, respectively. From birds that gave weak reactions to the MOMP with pre-inoculation plasma samples, strong reactions were detected by PID 7-10. Detection of the 97.4K, 60K, and the MOMP persisted for at least PID 142. Reactions were detected at variable times with several other bands, including those of 72K, 55K, 39K, and 38K (Fig. 3). Plasma from contact exposed birds (group 2) showed similar results, except reaction to the 97.4K protein was first detectable at PID 21-28 (Fig. 4). Prior to inoculation, all birds had no detectable antibodies against TT3 OMP bands. Weak reactions between the MOMP and pre-inoculation samples were occasionally seen.

Immunoblot analysis of synovial joint fluid

Synovial joint fluid was collected on the day of the last bleeding. Immunoblots using joint fluid samples (indicated as JF) are depicted in Figs. 3, 4. All birds had detectable IgG specific for the MOMP, 60K, and 97.4K bands. The IgG profiles from joint fluid samples were very similar to those obtained from plasma which were collected on the same PID.

Immunoblot analysis of plasma with chlamydiae

Antibody responses to heterologous chlamydial strains were examined. Distribution of antigenic determinants of VS1

and B577 recognized by antibodies from TT3-inoculated turkeys were similar to that of TT3 (Fig. 5). Bands at 60K and 97.4K were visible in both VS1 and B577 OMP immunoblots. The MOMP of TT3 showed a strong reaction while MOMPs of VS1 and B577 showed variable reactions with plasma from TT3-inoculated birds. The OMP profile of India ink-stained transblots of strains TT3, VS1, and B577 are included for reference.

Immunological cross-reactivity

Outer membrane proteins of TT3 were used for immunoblotting with sera from turkeys experimentally exposed to heterologous gram-negative bacteria. Turkey sera with antibodies against Escherichia coli (O78:K80:H9 or O2:K-:H6), Bordetella avium, Mycoplasma meleagridis, M. gallisepticum, M. synoviae, or Pasteurella multocida showed no or very faint (only to the MOMP) reaction with TT3 OMPs (data not shown). Also, gnotobiotic turkey sera showed no reaction to TT3 OMPs.

Effect of proteinase, trypsin, and periodate treatments on OMPs

The OMP-enriched preparation was treated with proteinase or sodium metaperiodate prior to SDS-PAGE analysis. Coomassie blue-stained SDS-polyacrylamide gel (Fig. 6) of OMPs and periodate-treated OMPs showed that the protein profile was unchanged, while the OMP profile of the proteinase-treated sample (Lane 3) was eliminated.

Immunoblot analysis of these gels showed detectable antigenic epitopes with the periodate-treated OMPs but not with the protease-treated sample (Fig. 7). No detectable bands were seen on trypsin-treated bands containing OMPs (Fig. 7). Proteinase treatment did not affect the broad, silver-stained, low-molecular-weight band of TT3 (Fig. 6).

FIG. 1. Outer membrane protein-enriched preparation extracted with sarkosyl from elementary bodies of Chlamydia psittaci TT3, stained with ruthenium red, sectioned, and examined with the electron microscope (A). Higher magnification (B) demonstrates outer membrane morphology. Membrane diameter is 8 nm and vesicle diameters are 0.2 to 0.4 um. Bars, 0.1 um.

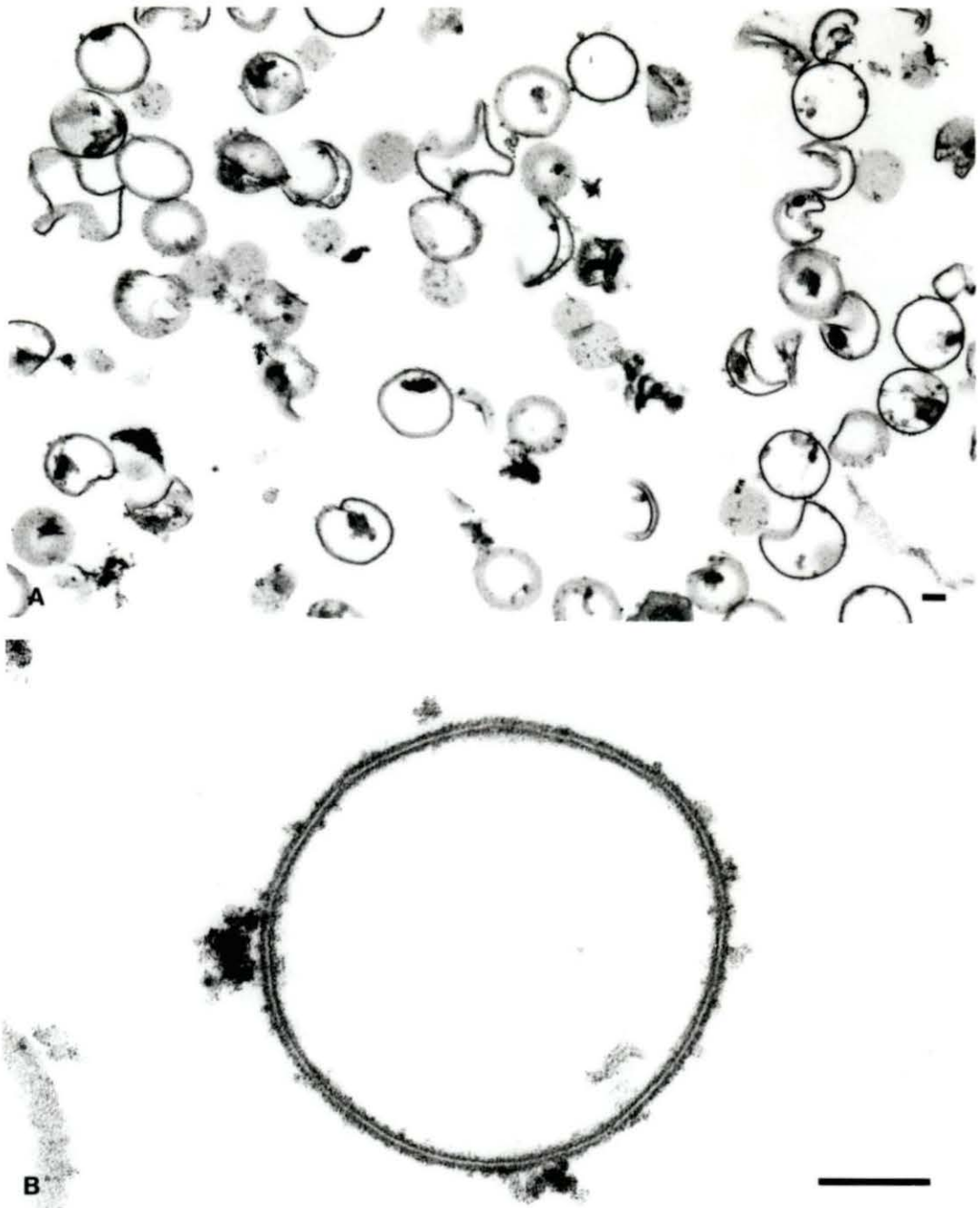

A
B

FIG. 2. SDS-PAGE of outer membrane protein-enriched preparation extracted from Chlamydia psittaci TT3. Elementary bodies were isolated through renografin, disrupted by ultrasound, and treated with sarkosyl before electrophoresis. Protein bands were visualized by staining with silver. Positions of the immunoreactive chlamydial proteins are indicated at the right, with apparent molecular weights (10^3). Positions of molecular weight (10^3) standards are on the left.

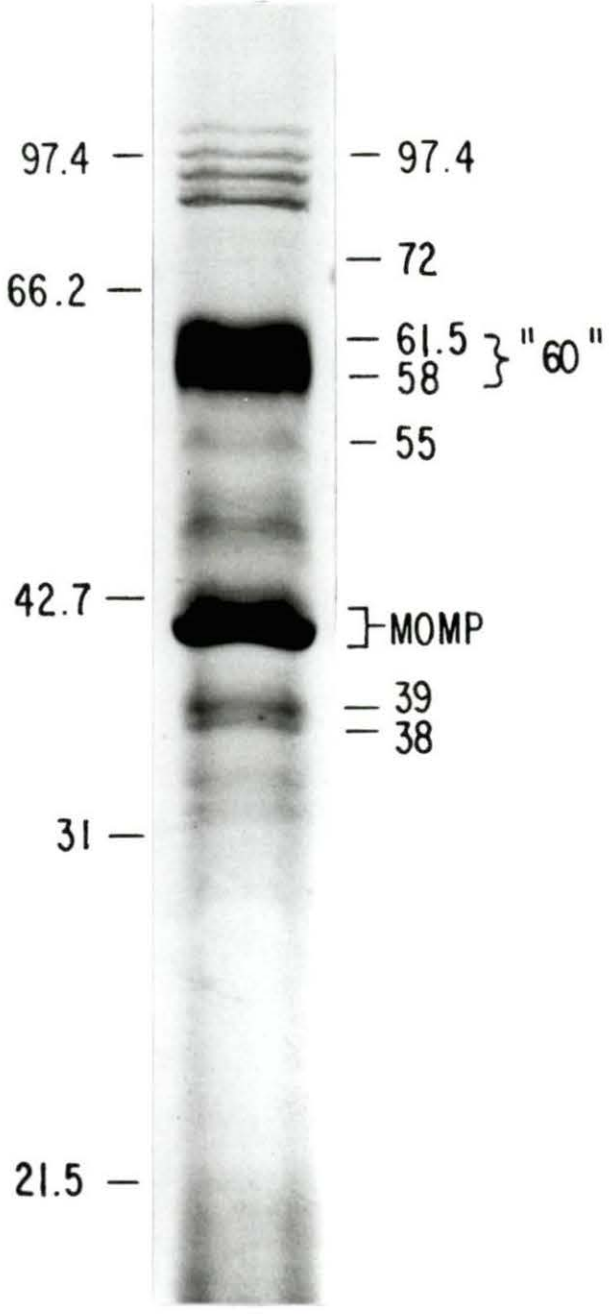

97.4
66.2
42.7
31
21.5
97.4
72
61.5
58
"60"
55
MOMP
39
38

FIG. 3. Transblot of outer membrane proteins (OMPs) of Chlamydia psittaci TT3 stained with India ink (A). Immunoblot analysis of OMPs using plasma obtained serially from two TT3-inoculated turkeys (B, C). Antibody reactivity was detected using biotinylated rabbit anti-turkey IgG (H+L) followed by avidin conjugated to alkaline phosphatase with 4-chloro-1-naphthol as the substrate. Days which individual plasma samples were collected are shown beneath each lane. The 97.4K, 60K, and MOMP are labeled on the right. JF = joint fluid; MOMP = major OMP.

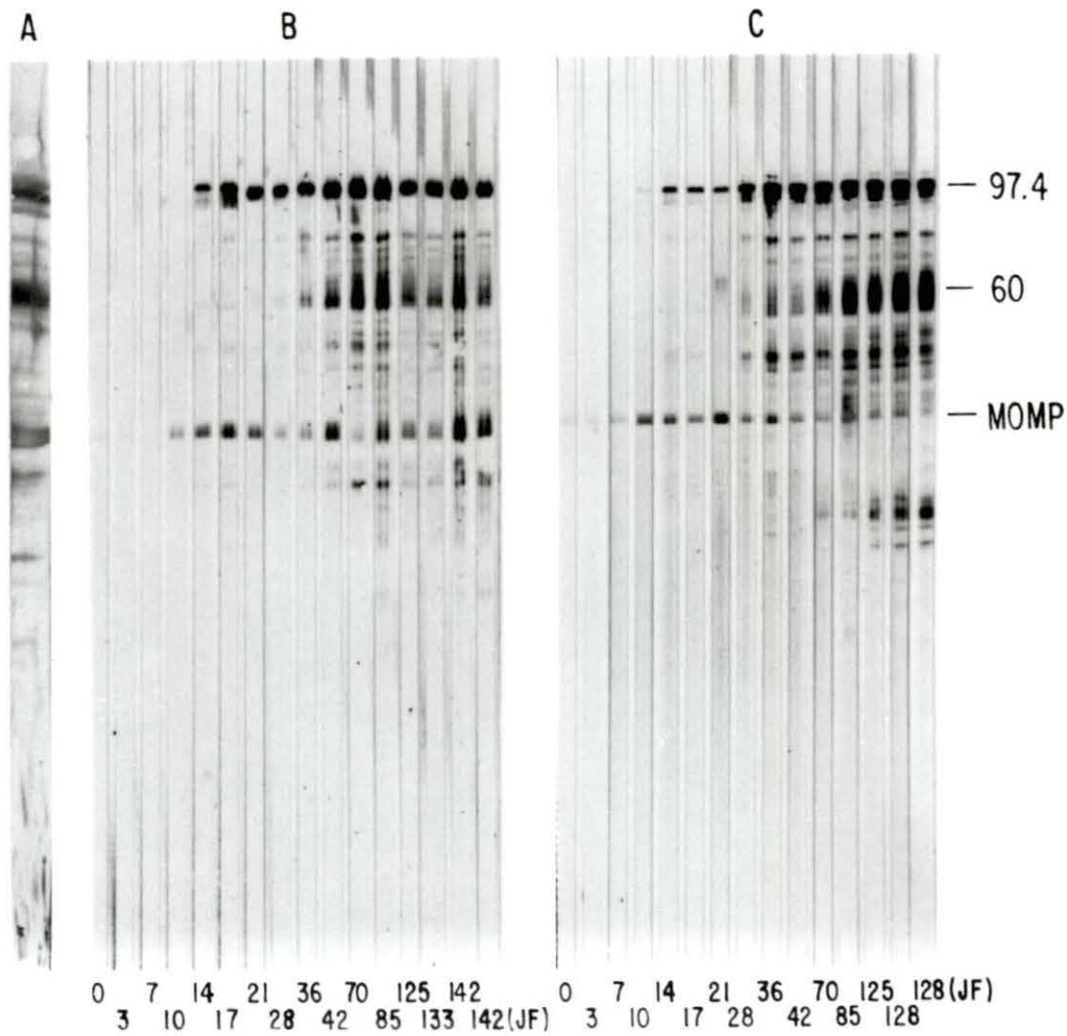

A
B
C
— 97.4
— 60
— MOMP
0 7 14 21 36 70 125 142
3 10 17 28 42 85 133 142(JF)
0 7 14 21 36 70 125 128(JF)
3 10 17 28 42 85 128

FIG. 4. India ink-stained transblot of outer membrane proteins (OMPs) of Chlamydia psittaci TT3 was used to locate protein bands (A). Immunoblots showing reactions of plasma obtained serially from two TT3 contact-exposed turkeys (B, C) with OMPs of TT3. Immunocomplexes were detected using biotinylated rabbit anti-turkey IgG (H+L) followed by avidin conjugated to alkaline phosphatase with 4-chloro-1-naphthol as the substrate. Days which individual plasma samples were collected are shown beneath each lane. The 97.4K, 60K, and MOMP are labeled on the right. JF = joint fluid; MOMP = major OMP.

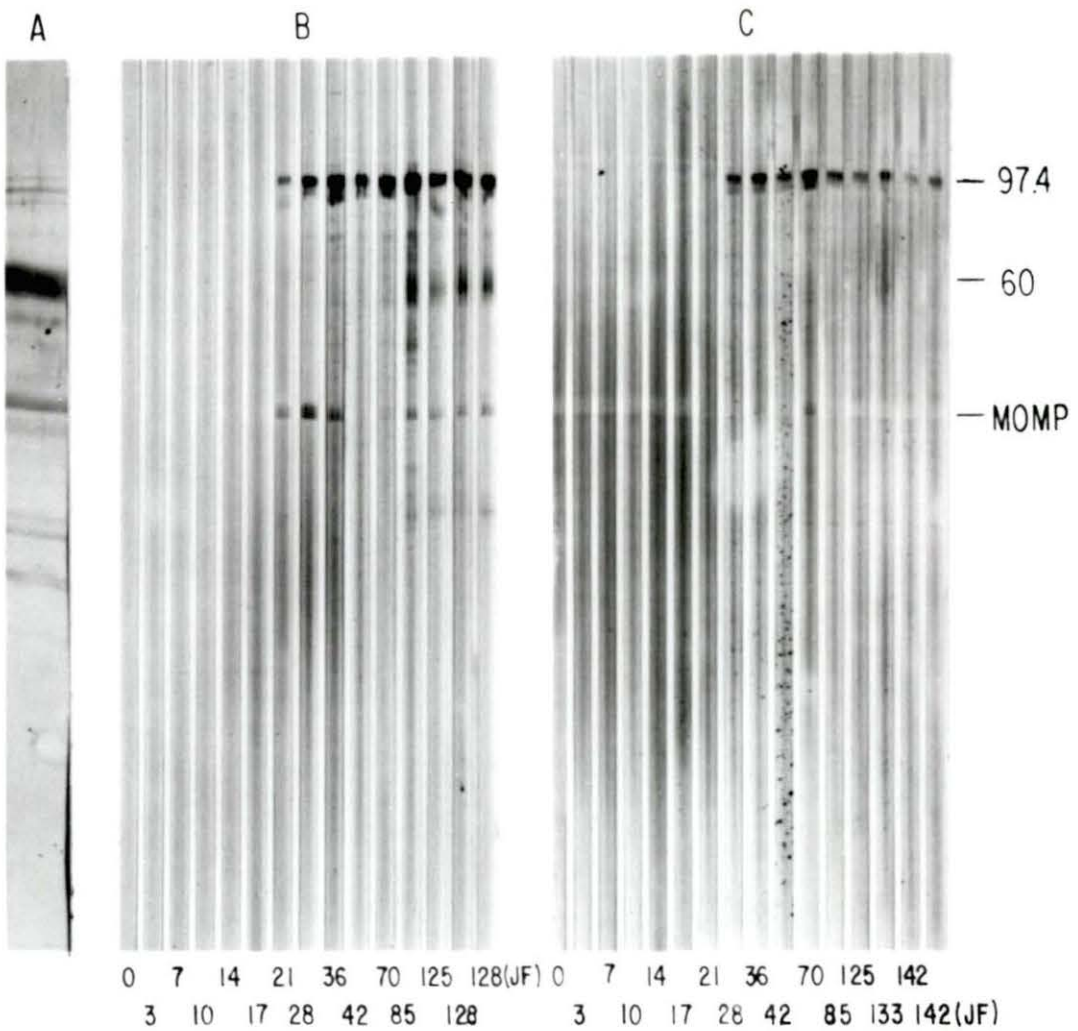

A
B
C
97.4
60
MOMP
0 7 14 21 36 70 125 128(JF)
3 10 17 28 42 85 128
0 7 14 21 36 70 125 142
3 10 17 28 42 85 133 142(JF)

FIG. 5. Transblots of outer membrane protein-enriched preparations extracted from elementary bodies of Chlamydia psittaci TT3, VS1, and B577 stained with India ink (A). Immunoblots showing reactions of plasma obtained from a TT3-inoculated turkey with each transblot (B). Antibody reactivity was detected using biotinylated rabbit anti-turkey IgG (H+L) followed by avidin conjugated to alkaline phosphatase with 4-chloro-1-naphthol as the substrate. Strain identities of transblots are indicated at top. The 97.4K, 60K, and major outer membrane protein (MOMP) are indicated at the left.

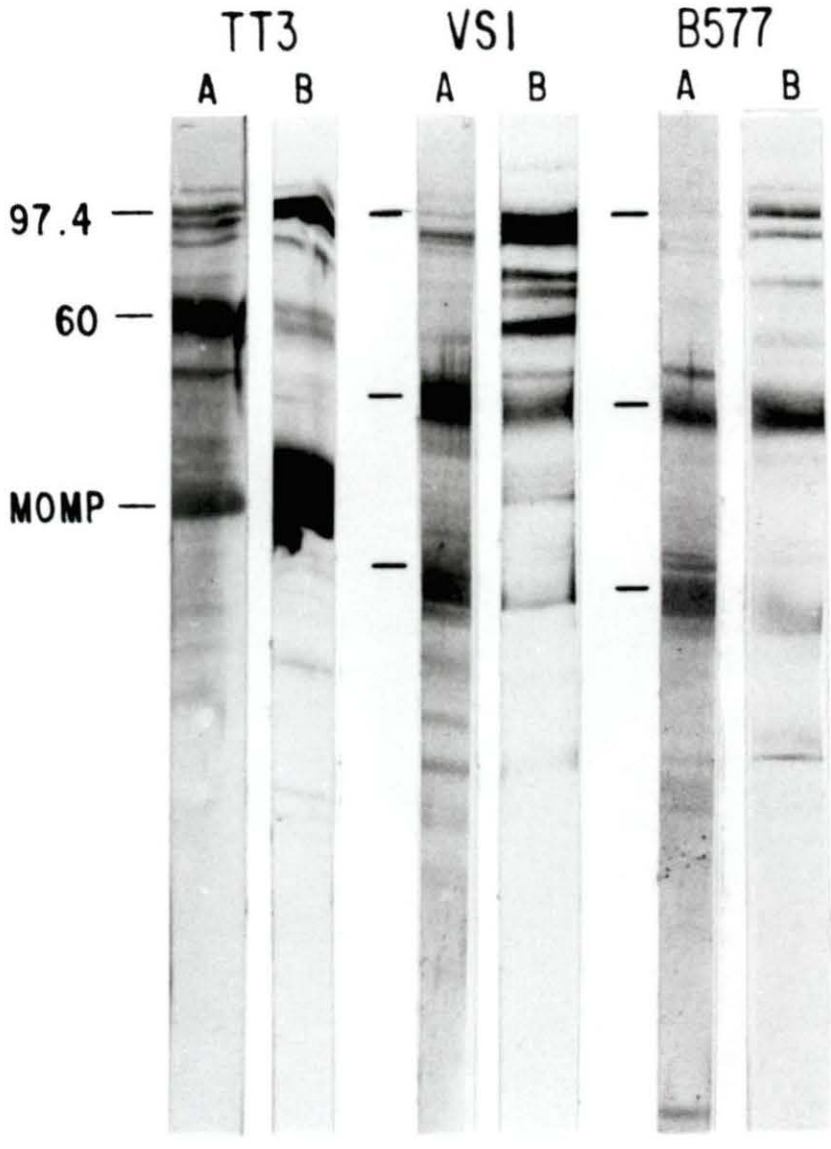

TT3
VSI
B577
A
B
A
B
A
B
97.4
60
MOMP

FIG. 6. SDS-PAGE analysis of outer membrane protein-enriched preparation extracted from Chlamydia psittaci TT3 stained for protein with Coomassie blue (A) or for lipopolysaccharide with silver (B). Elementary bodies were isolated through renografin, disrupted by ultrasound, and treated with sarkosyl before electrophoresis. Sarkosyl-insoluble outer membrane proteins were untreated (lane 1), treated with periodate (lane 2), or treated with proteinase K (lane 3) prior to electrophoresis. Positions of molecular weight (10^3) standards are indicated.

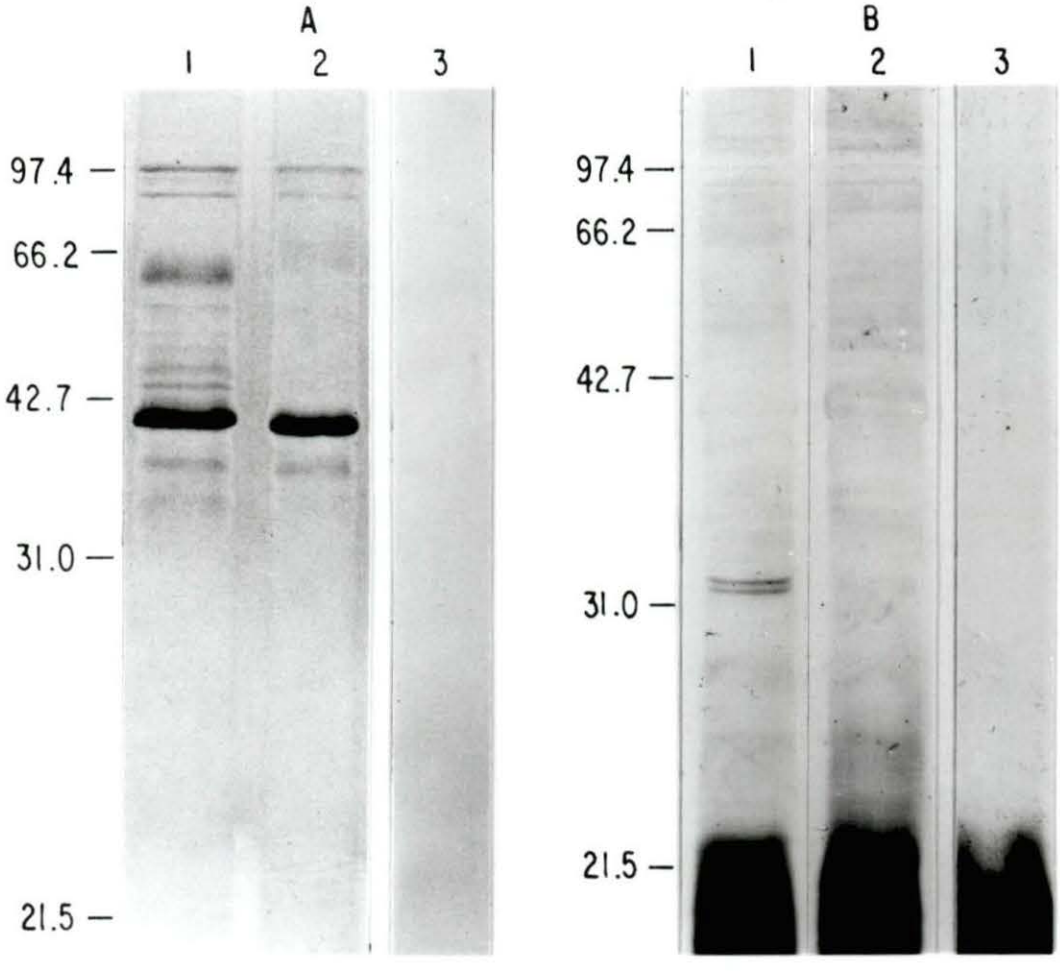
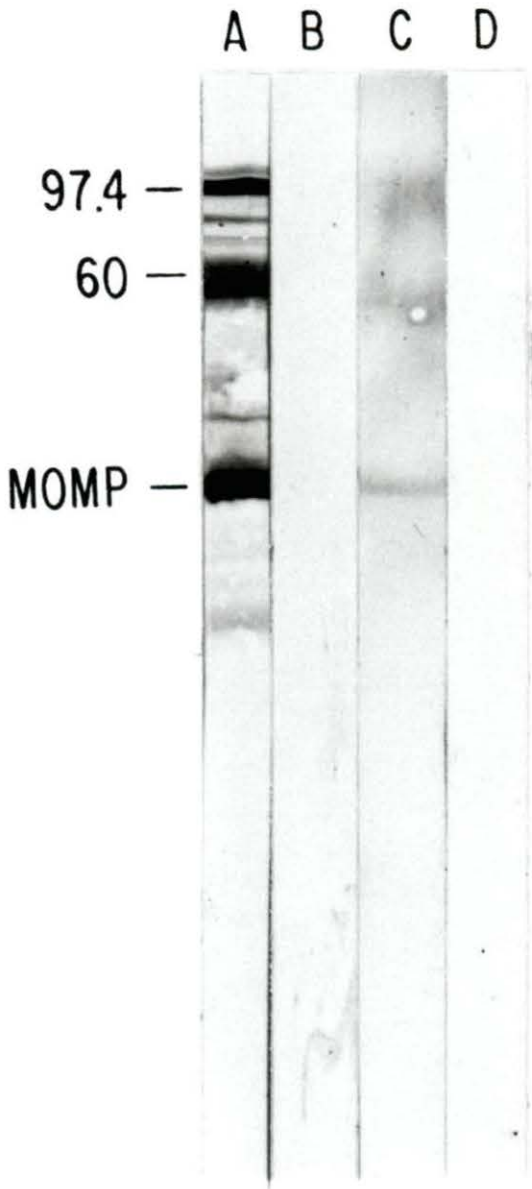

A
1
2
3
97.4
66.2
42.7
31.0
21.5
B
1
2
3
97.4
66.2
42.7
31.0
21.5

FIG. 7. Immunoblot analysis of outer membrane protein-enriched preparation extracted from Chlamydia psittaci TT3 using plasma from TT3-inoculated turkey. Prior to SDS-PAGE separation and electrophoretic transfer to nitrocellulose, samples were untreated (lane A), treated with proteinase K (lane B), or treated with periodate (lane C). Lane D represents trypsin treatment of transblot of outer membrane protein-enriched preparation before immunoblotting. Location of the 97.4K, 60K, and major outer membrane protein (MOMP) are marked.


A
B
C
D
97.4
60
MOMP

DISCUSSION

This study investigated humoral antibody (IgG) responses of turkeys to specific chlamydial proteins of surface origin, using immunoblotting techniques to follow the time course of antibody production during infection. Antibody persistence to PID 142 was demonstrated. Antigenic analysis of OMPs was employed to determine individual outer membrane components which elicit antibody response in turkeys experimentally exposed to TT3, a virulent avian (turkey) strain of C. psittaci.

A predominance of antibody to cell-surface-exposed antigenic determinants of high-molecular-weight (97.4K) OMPs was noted: this indicates that these relatively large proteins are more immunogenic in turkeys than are other C. psittaci proteins, perhaps because they are more accessible to the immune system of the turkey. Specifically, the 97.4K protein was recognized in vivo during infection, and was the first OMP to which the turkey produced appreciable quantities of IgG.

Immunoblotting proved to be a reliable method for the detection of C. psittaci infections in turkeys, which are often difficult to detect because of the organism's obligate intracellular life cycle. Since C. psittaci is an invasive infection, circulating antibodies against OMPs reflect the humoral immune response to this organism. In plasma and

synovial joint fluid from experimentally-exposed birds, immunoblotting was extremely sensitive and specific in the specimens tested.

Horizontal transmission of C. psittaci between birds was demonstrated by isolation of TT3 from tracheal and cloacal swabs from pen-contact birds (group 2) which were housed with TT3-inoculated birds (group 1). In addition, seroconversion of contact-exposed birds was demonstrated by immunoblotting. Plasma from these birds showed detectable IgG to the 97.4K protein at PID 21-28 in comparison with PID 10-14 for birds inoculated with TT3. The antibody response was identical other than the delay due to time of infection.

Immunoblotting experiments by Newhall et al. (108) have demonstrated that antibody to the 60K and 62K proteins were dominant in the majority of sera from individuals with genital infections of C. trachomatis. They also found that the MOMP was generally only weakly reactive despite the predominance of this protein in the EB cell wall. Turkey antibody reactivity with the 60K and MOMP proteins were also noted. However, detection of antibodies to these proteins was variable between birds during the time course of antibody production.

McClenaghan et al. (94) have shown that several proteins reacted with the sera of sheep vaccinated with the ovine abortion strain of C. psittaci. Dominance of three relatively high molecular weight polypeptides (78-90K) gave

the most marked antibody response. In turkeys, we also noted that a protein of similar molecular weight (i.e., the 97.4K band) showed the most pronounced reactions. Differences in molecular weight of proteins between studies may be attributed to antigen separation techniques. Interestingly, there was only a slight reaction to MOMP, which was similar to the reactions demonstrated when sera of humans with genital infections of C. trachomatis were tested against protein antigens of C. trachomatis (108).

The antibody response to specific chlamydial antigens during ocular infection has been described by immunoblot analysis of sera and tears from monkeys inoculated with a human strain of C. trachomatis (36). In tears from monkeys with acute C. trachomatis serovar B conjunctivitis, IgA specific to MOMP appeared approximately PID 14 and were still evident at PID 56. Antibodies to 60K and 54K proteins developed between PID 21 and 28. There was great variability in presence and intensity of response to other antigens. As shown in our study, the first detectable antibody response was to the 97.4K protein at approximately PID 10. Antibody reactions with the 60K and MOMP were variable in intensity and were first detected between PID 14-42 and PID 7-70, respectively.

Guinea pigs inoculated intravaginally with a GPIC strain have been used as a model for human genital infections (14). Immunoglobulins G and IgA were detected by immunoblotting in

ocular secretions soon following a primary infection in the genital tract (ocular infection absent). Serum IgG specific to the MOMP appeared approximately PID 12, peaked at PID 20-30, decreased slightly between PID 50 and 90, and remained relatively unchanged as long as 825 days. Antibodies to the 61K protein developed approximately the same time as the MOMP but continued to increase while anti-MOMP decreased. Immunoglobulin A from genital secretions appeared on approximately PID 20 for both the 61K and MOMP, but was minimal by PID 30. Antibody responses to other membrane components and their various patterns of waxing and waning were noted during the observation period. Plasma from TT3-infected turkeys contained antibodies to the 97.4K, 60K, and MOMP which were detected for at least PID 142.

The C. psittaci strains TT3 (turkey), VS1 (psittacine), and B577 (ovine abortion) used in this study are heterologous in their antigenicity: on the one hand, they have common epitopes, detected in this study with turkey immune serum by immunoblotting with OMPs of the different strains; on the other hand, they have been shown to have specific epitopes recognized by monoclonal antibodies using indirect fluorescent antibody staining (5). The OMP-enriched preparation from TT3 did not contain antigens which were detected by antibodies from turkeys exposed to Escherichia coli, Bordetella avium, Mycoplasma meleagridis, M. gallisepticum, M. synoviae, or Pasteurella multocida.

Also, no reaction to TT3 OMPs was detected using gnotobiotic turkey sera.

By enzyme and chemical treatments, the antigens against which the antibody response was directed appear to be mainly toward the protein components of the OMP-enriched preparation. Proteinase digestion and trypsinization (but not periodate oxidation) of the OMP-enriched preparation removed reactivity as demonstrated by immunoblot analysis. The Coomassie blue-stained SDS-polyacrylamide gel showed that the OMP profile was not affected by the periodate oxidation but was eliminated by proteinase digestion. The inability to detect antibodies against LPS could be due to the detergent used in the immunoblotting procedure: it has been reported that chlamydial LPS binds very poorly to nitrocellulose when buffers containing detergents are used (33). These properties demonstrate that the antigens recognized in this system are protein in nature.

The immunoblot analysis of plasma from turkeys may aid in the diagnosis of chlamydiosis in field outbreaks. Diagnosis by isolation of C. psittaci from field specimens is difficult. Demonstration of chlamydial inclusions in tissue cell culture is the standard for laboratory diagnosis of chlamydiae but is time-consuming and therefore of limited value for the timely diagnosis of chlamydial outbreaks in turkey flocks. Swab type, transport medium, transport conditions, and storage of specimens are important for the

success of subsequent isolation attempts for diagnosis of avian chlamydiosis (150). Prior antibiotic treatment of birds can prevent detection of chlamydia. "Cryptic" forms of chlamydia occur in vitro (103). These forms may affect several diagnostic procedures: 1) isolation of C. psittaci from tissues or body fluids; 2) detection of antigens by ELISA (38), immune dot blot (95), monoclonal antibody immunofluorescence (150), and immunoperoxidase staining (98) techniques; or 3) detection of nucleic acids by in situ hybridization (74) and DNA spot hybridization (150).

On the other hand, immunoblotting techniques using chlamydial antigens have demonstrated the presence of circulating antibodies against chlamydial membrane components in humans (108), in sheep (94), in monkeys (36), and in guinea pigs (14). Results of this study has also demonstrated that immunoblot analysis of plasma from turkeys infected with a virulent turkey strain of C. psittaci can be used to detect antibodies against chlamydial antigens, particularly to the 97.4K OMP.

Information gained from this investigation will aid in determining the status of turkeys in a flock. Carrier birds or birds with recently acquired C. psittaci infections would be detected by testing their serum by immunoblotting with OMPs of TT3. A seronegative bird would indicate no previous contact with chlamydiae, whereas a seropositive bird could be a potential carrier.

In conclusion, the humoral antibody response in turkeys experimentally exposed to C. psittaci TT3 was shown to be active in the production of specific antibodies to OMPs of chlamydiae. The 97.4K, 60K, and MOMP were the predominant antigens recognized by these antibodies. Antibodies do have both neutralizing (32, 35, 121) and opsonizing (164, 165) functions against chlamydiae in vitro. The data presented from this investigation warrant further study of high-molecular-weight proteins for possible candidates in vaccine development as protective antigens and also in diagnostic evaluation of avian chlamydiosis. In conjunction with other studies such as surface labelling of EBs and immunoprecipitation techniques, the described immunoblotting techniques should make a valuable contribution to the understanding the turkey's response to chlamydial infection.

PART II. IMMUNOCHEMICAL PROFILES OF OUTER MEMBRANE PROTEINS OF STRAINS FROM AVIAN AND MAMMALIAN *CHLAMYDIA PSITTACI*

ABSTRACT

Immunochemical properties of outer membrane proteins (OMPs) from avian (turkey/TT3 and psittacine/VS1) and mammalian (ovine abortion/B577) strains of Chlamydia psittaci were compared using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting with sera from turkeys exposed to an avian or mammalian strain. The objective was to demonstrate antibody reactions with antigens from specific strains of C. psittaci using sera from turkeys experimentally exposed to avian or mammalian chlamydiae. Among the strains, the major OMP (MOMP) and several bands (85-105K) had variable electrophoretic patterns and immunological reactions. Sera from turkeys exposed to an avian or mammalian strain could be separated on the basis of immunoreactive patterns with OMPs of homologous and heterologous strains. The 97.4K OMP of avian and mammalian strains reacted with sera from birds exposed to avian but not to mammalian C. psittaci. Fewer bands and often weaker reactions were detected by immunoblotting of sera from TT3- and VS1-inoculated birds with the heterologous avian strain. Also, these sera reacted with the MOMP of B577 and homologous strains, but not consistently with the heterologous avian strain. In contrast, reactions between sera from B577-inoculated birds with the MOMP of each strain were not detected or were weak. Differences described in this study between avian and

mammalian strains support their differentiation into separate biovars and may relate to their differences in pathogenicity.

INTRODUCTION

Specific strains of Chlamydia psittaci are associated with distinct pathological syndromes of chlamydiosis in turkeys (16, 17, 112, 113, 114, 117, 118, 119, 149). Highly virulent strains cause acute epornitics in which 5 to 30% of affected birds die and also may infect humans associated with the processing of these turkeys. Chlamydial epornitics of mild virulence occasionally occur among turkey flocks, but no human infections are associated with these outbreaks (119).

Avian C. psittaci can be categorized by restriction endonuclease (3, 4) and monoclonal antibody (5) analyses into groups (virulent turkey or psittacine-like) which are distinct from mammalian groups. Also, they exhibit differences in terms of natural and experimental pathogenicity for wild, domestic, and laboratory animals (16, 17, 112, 113, 114, 117, 118, 119, 149). Chlamydia psittaci strains TT3 (virulent turkey), VS1 (parrot), and B577 (ovine abortion) are representative isolates of the respective groups, as defined by Andersen's restriction endonuclease analysis (3) and monoclonal antibody specificity studies (5). Differences in virulence for turkeys between these strains have been described (149). Turkeys infected with TT3 were lethargic; and had decreased body weight, airsacculitis, bronchopneumonia, and severe pericarditis. Birds infected with VS1 had severe airsacculitis and bronchopneumonia, but a

low incidence of pericarditis. Only mild peribronchial pneumonia was detected in B577-inoculated turkeys.

The purpose of this investigation was to demonstrate antibody reactions with antigens from specific strains of C. psittaci using sera from turkeys experimentally exposed to avian or mammalian chlamydiae. Protein profiles and antigens of chlamydial outer membrane proteins (OMPs) were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis. Immunoblots were conducted using sera from turkeys experimentally exposed to C. psittaci TT3 (virulent turkey), VS1 (psittacine), or B577 (ovine abortion) strains.

MATERIALS AND METHODS

Chlamydiae and growth conditions

The chlamydial strains used in this study are listed in Table 1. Cultures of each strain were obtained from the stock collection at the National Animal Disease Center, Ames, Iowa. Chlamydiae were propagated 2 to 5 days at 37° C in Vero cells (American Type Culture Collection, Rockville, MD) in the presence of cyclohexamide (Sigma, St. Louis, MO) (0.5 mg/ml). Vero cells were maintained in F15 Eagle's Minimal Essential Medium (pH 7.2) containing Earl's Balanced Salts (Gibco, Grand Island, NY), 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; Calbiochem-Behring, La Jolla, CA), 2 mM L-glutamine (Gibco), 100 mM glucose, 5% fetal bovine serum (inactivated by heating at 56° C for 30 min), 20 ug/ml gentamicin sulfate (Scherring Corp., Kenilworth, NJ), and 4 ug/ml amphotericin B (E. R. Squibb & Sons, Inc., Princeton, NJ). Cultures were examined at 24 h intervals to detect cytopathic effects or cell necrosis (sloughing). Chlamydia were harvested when greater than 20% of tissue culture cells had died or showed cytopathic effects. Vero cells were removed by centrifugation (500 x g for 10 min). The supernatant was collected and then centrifuged (10,000 x g for 1 h). The pellet containing chlamydiae was suspended in Bovarnick's Buffer (23) and then stored at -80° C until needed.

TABLE 1. Source of strains of Chlamydia psittaci

Strain	Origin	Yr Isolated	Host	Disease	Reference or Source
TT3	Texas	1975	Turkey	Ornithosis	Page[a]
VS1	Georgia	1985	Parrot	Psittacosis	NVSL[b]
B577	Utah	1962	Sheep	Abortion	142

[a] Isolated by L. Page from 1974-1976 Texas outbreak (119).
[b] National Veterinary Service Laboratories, Ames, Iowa (5).

Turkeys

Twelve-week-old broad-breasted white turkeys (nonvaccinated) were inoculated intratracheally with 10 mM NaH_2PO_4-0.9% NaCl, pH 7.2 (PBS) (control group) or PBS containing approximately 10^4 IFU of either C. psittaci TT3 (group 1), VS1 (group 2), or B577 (group 3). Birds were separated by groups and housed on litter in isolation rooms. Antibiotic-free food and water were provided ad libitum. Birds were observed for clinical signs. Tracheal and cloacal swabs were collected for bacterial isolation before and on alternate days for two weeks then weekly for 10 weeks post-inoculation day (PID). Serum samples from birds of each group were also collected at same time intervals.

Elementary bodies

Elementary bodies (EBs) of each strain were isolated by density gradient centrifugations in Renografin (34). Chlamydiae frozen in Bovarnick's Buffer were thawed and then centrifuged (50,000 x g for 20 min) through 35% Renografin-76 (R76; E. R. Squibb & Sons, Inc., Princeton, New Jersey). The pellet was suspended in H_2O and layered on a 35-44-51% R76 step gradient. The gradient was centrifuged at 69,000 x g for 1.5 h at 4° C. The band containing EBs was collected and washed in H_2O by centrifugation at 50,000 x g for 20 min. The pellet was suspended in 20 mM Tris , pH 7.6. Purity of EBs was determined by electron microscopy.

Outer membrane proteins

Elementary bodies were sonicated for 3 min at 30% output power (Heat systems-Ultrasonics, Inc., Farmingdale, NY). Whole cells were then removed by centrifugation (4,000 x g for 15 min) and the supernatant containing broken chlamydial cells was mixed (1:1) with two percent sodium N-lauroyl sarcosinate (Sigma) in 20 mM Tris, pH 7.6, for 30 min at 37° C. Sarkosyl-insoluble fraction was pelleted by centrifugation at 105,000 x g for 1 h at 5° C. The pellet was suspended in 10 mM HEPES, pH 7.2, containing 150 mM NaCl, 10 mM $MgCl_2$, 50 ug of DNase I (Sigma), and 100 ug of RNase I-A per ml (Sigma) per ml (22). This suspension was incubated 30 min at 37° C, and then centrifuged at 105,000 x g for 1 h

at 4° C. The pellet containing OMPs was resuspended in H_2O and stored at -80° C until needed. Protein concentration was determined by Coomassie brilliant blue (26) using bovine serum albumin as a standard.

Electron microscopy

Purity of EB preparations were determined by thin sectioning and negative staining techniques (27, 71). Elementary bodies were fixed 3 h with 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer (ph 7.4), stained 2 h in 1% osmium tetroxide, dehydrated in graded ethanols and propylene oxide, and embedded in Medcast (Ted Pella, Inc., Tustin, CA). Thin sections (90 nm) were stained with lead citrate (123) and uranyl acetate (140).

Chlamydiae were stained with neutralized phosphotungstic acid and applied to coated copper grids using a nebulizer (27). All preparations were examined using a Philips 410 electron microscope (Philips Electronic Institute Inc., Mahwah, NJ) at an accelerating voltage of 60 kV.

Electrophoresis

Proteins were separated by SDS-PAGE with the two buffer system of Laemmli (84). Outer membrane protein-enriched preparations were heated (100° C) for 5 min in treatment buffer (2% sodium lauryl sulfate, 10% glycerol, 5% 2-mercaptoethanol, 0.003% bromphenol blue in 62.5 mM Tris [pH

6.8]) before electrophoresis. Vertical slab gels (LKB-Produkter AB, Bromma, Sweden) contained 15 to 20 ug of protein per lane or preparatory slab gels were loaded with 150 ug of protein. Gels were run 1.5 h at a constant current of 35 mA and then 2-3 h at 100 mA. Protein bands were visualized by staining with Coomassie brilliant blue R250 (Bio-Rad). Molecular weight for each band was determined from a plot of mobility versus log 10 of the mw of protein standards (Bio-Rad).

Immunoblotting

Detection of antibodies against OMP antigens of each strain was done according to the method of Towbin et al. (151) with modifications. Proteins from each strain were separated by SDS-PAGE (84) and electrophoretically transferred from the gel to nitrocellulose (0.45-um pore size; Schleicher & Schuell, Inc., Kene, NH). Transfers were done using a Bio-Rad Transblot cell (Bio-Rad) at 0.9 A for 20 min at 5° C in buffer containing 25 mM Tris, 192 mM glycine, 0.1% sodium dodecyl sulfate, and 20% (vol/vol) methanol. Transblots were blocked by immersion in PBS containing 0.05% Tween 20 (TPBS). The blots were cut into strips (4mm) and incubated 1 h with serum samples (1:100) from turkeys infected with avian or mammalian C. psittaci. Sera from six to ten birds of each exposure group were evaluated by immunoblotting. Detection of immunocomplexes used

biotinylated rabbit anti-turkey IgG (Zymed Laboratories, San Francisco, CA) followed by avidin conjugated to alkaline phosphatase with 4-chloro-1-naphthol (Bio-Rad) as the substrate. Nitrocellulose strips of each transblot were stained with Pelikan Fount India Ink (Pelikan AG, Hanover, Federal Republic of Germany) and used to determine efficiency of transfers and protein specificity of the immunoblots (64).

Major OMP- or lipopolysaccharide (LPS)-genus specific monoclonal antibodies (Chemicon International, Los Angeles, CA) were used as probes to identify the MOMP and the distribution of LPS molecules among the OMPs on the nitrocellulose.

RESULTS

Characterization of OMP profiles

Phenotypic differences of OMPs among strains of C. psittaci were studied by SDS-PAGE. The electrophoretic patterns of sarkosyl-insoluble OMP-enriched preparations revealed substantial heterogeneity as well as homogeneity between TT3, VS1, and B577. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed qualitative as well as quantitative differences in protein bands. A major band (i.e., MOMP) and 10 to 20 less intensely Coomassie blue-stained bands were resolved for each strain on SDS-PAGE gels (Fig. 1). The OMP profiles were similar; however, differences were apparent in the migration of the major bands (MOMP) from the three strains. The MOMP varied from 39,000- to 42,000-molecular weight. Also, differences in migration were seen in bands of relatively high molecular weights (85-105K). The MOMPs were 41.5K, 41K, and 39.5K in preparations from VS1, TT3, and B577, respectively (Fig. 1). Numerous less intensely stained bands (35K to 39K, 58k to 62K, and 72K to 78K) were evident in the OMP-enriched preparations from all strains.

Protein profiles of TT3, VS1, and B577 EBs disrupted by sonication are shown in Fig. 2. A major band (i.e., MOMP) intensely stained with Coomassie blue in the 39,000- to 42,000-molecular weight region was apparent for each strain

on SDS-PAGE gels. Numerous bands were separated in these preparations. Some of them included the bands which were present in sarkosyl-insoluble OMP-enriched preparations (Fig. 2, lanes 4, 5).

Immunological strain specificity

Antibody responses against homologous and heterologous strains were detected by immunoblotting. Post-exposure (day 70) sera from turkeys inoculated with PBS, TT3, VS1, or B577 were tested with OMP-enriched preparations from TT3, VS1, and B577 (Fig. 3). Pre- and post-exposure sera from turkeys exposed to TT3, VS1, or to B577 were also tested with homologous antigen preparations. Weak reactions between the MOMP and pre-inoculation samples were occasionally seen. Antibodies against other OMPs of TT3, VS1, and B577 were not detected prior to inoculation and from PBS-inoculated birds (Fig. 3, lane 4).

Antibodies in sera from TT3-inoculated turkeys reacted with OMPs of homologous and heterologous strains. Distribution of VS1 and B577 OMPs which reacted with antibodies from TT3-inoculated turkeys were similar to those of TT3 (Fig. 3, lane 1). Bands at 48K, 55K, 60K, 72K, 78K, and 97.4K were visible in immunoblots of OMPs from TT3, VS1 and B577. Strong reactions to the 97.4K band were visible in immunoblots of OMPs from TT3, VS1, and B577. The MOMP of TT3 strongly reacted with sera from TT3-inoculated birds. In

contrast, reactions were less intense to the MOMP of B577 and were variable to the MOMP of VS1. Variable reactions of numerous reactive bands (35K to 40K, 55K to 65K, and 85K to 110K) from the OMP-enriched preparations from TT3, VS1, and B577 were detected.

Reaction patterns of sera from VS1-inoculated turkeys with homologous antigens (Fig. 3, lane 2) were similar to those of sera from TT3-inoculated birds with their homologous antigens. Bands at 48K, 55K, 60K, 72K, 78K, and 97.4K were visible in immunoblots of OMPs from both VS1 and B577. Variable reactions with other bands (35K to 40K, 55K to 65K, and 85K to 110K) from OMP-enriched preparations of VS1 and B577 were also detected. The MOMP of VS1 reacted with sera from VS1-inoculated birds. In contrast, reactions were less intense to the MOMP of B577 and were variable to the MOMP of TT3. Weak reactions with MOMP, 55K, and 60K bands of TT3 were occasionally detected when using sera from VS1-inoculated birds. Strong reactions with the 97.4K band were visible in immunoblots of OMPs from VS1, TT3, and B577. Of particular note is that all sera from both VS1- and TT3-inoculated birds showed antibodies reacting with the 97.4K band of both homologous and heterologous OMPs.

Sera from B577-inoculated turkeys showed very little immunoreactivity with OMPs from TT3, VS1, and B577 (Fig. 2, lane 3). Weak reactions were detected to the MOMP of homologous antigens. One serum sample from one of six B577-

inoculated birds weakly reacted with MOMP of the heterologous antigens TT3 and VS1. Also, a different serum sample from one of the six B577-inoculated birds weakly reacted with the 60K band from TT3, VS1, and B577.

FIG. 1. SDS-PAGE of sarkosyl-insoluble outer membrane protein-enriched preparations extracted from elementary bodies of Chlamydia psittaci TT3 (lane 2), VS1 (lane 3), and B577 (lane 4). Protein bands were visualized by staining with Coomassie blue. Elementary bodies were isolated through renografin, disrupted by ultrasound, and treated with sarkosyl before electrophoresis. The region of the major outer membrane protein (MOMP) and molecular weight (10^3) of standards (lane 1) are indicated.

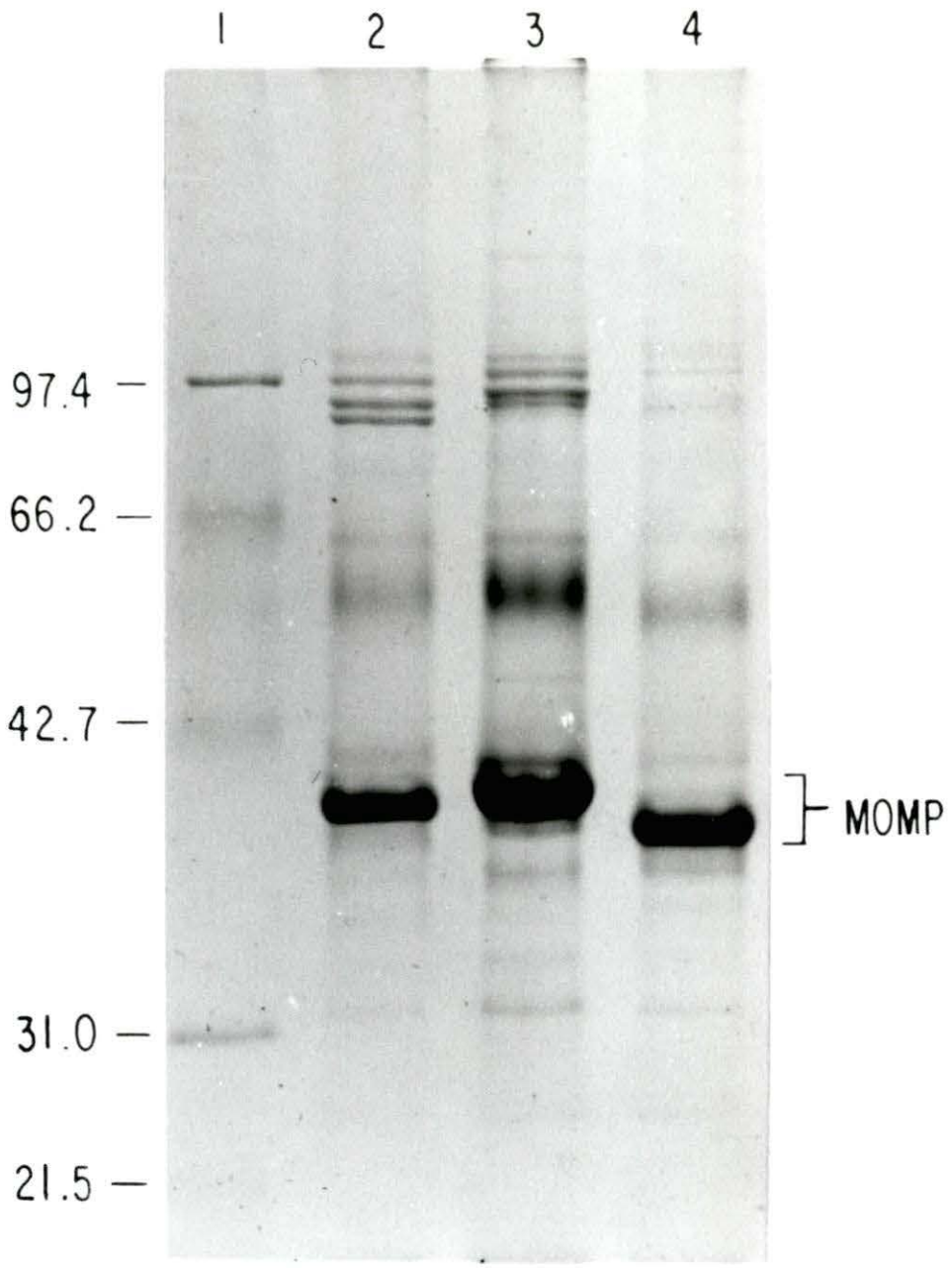

1
2
3
4
97.4
66.2
42.7
31.0
21.5
MOMP

FIG. 2. SDS-PAGE migration patterns of sonicated elementary bodies of Chlamydia psittaci B577 (lane 2), VS1 (lane 3), and TT3 (lane 4). Lane 5 contains sarkosyl-insoluble outer membrane protein-enriched preparation extracted from TT3. Protein bands were visualized by staining with Coomassie blue. The region of the major outer membrane protein (MOMP) and molecular weights (10^3) of standards (lane 1) are indicated.

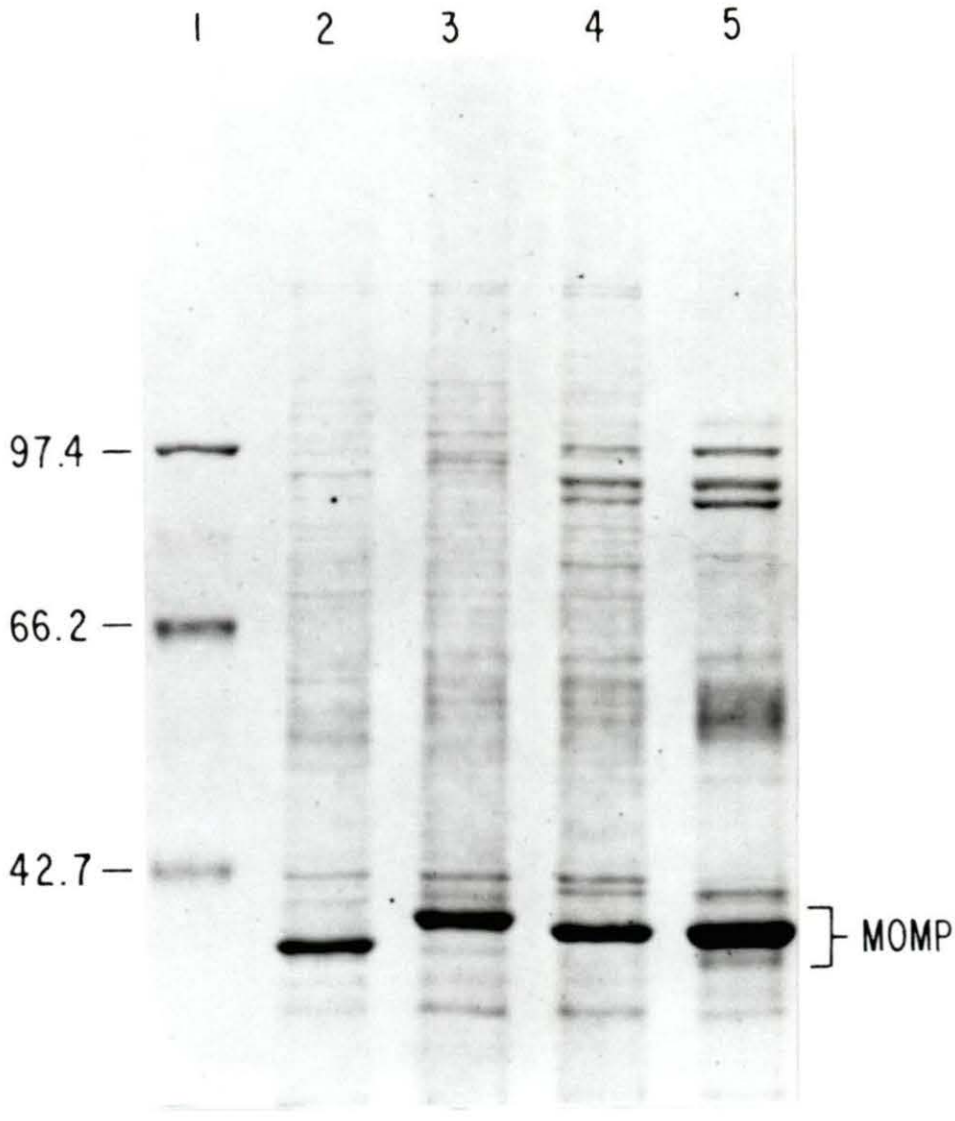
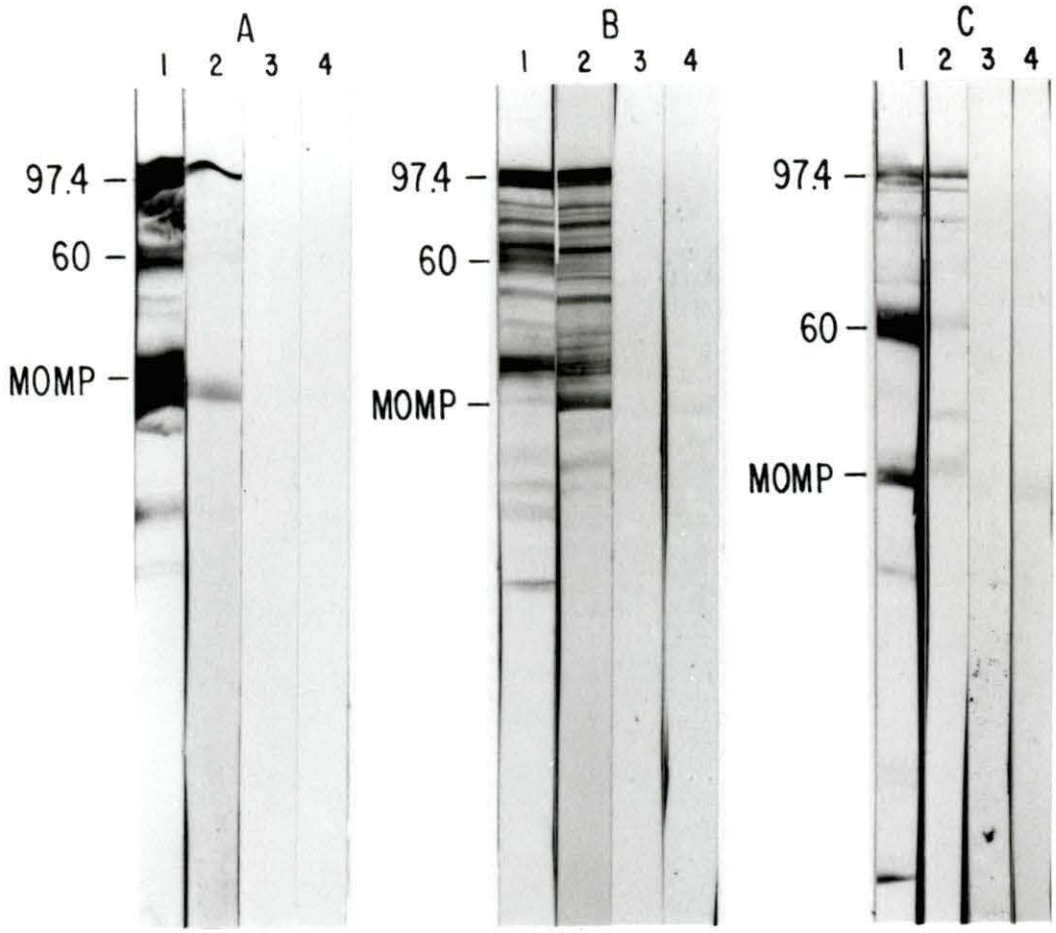

1
2
3
4
5
97.4
66.2
42.7
MOMP

FIG. 3. Immunoblots using outer membrane protein-enriched preparations extracted from Chlamydia psittaci TT3 (A), VS1 (B), and B577 (C) with serum from turkeys inoculated with C. psittaci TT3 (lanes 1), VS1 (lanes 2), B577 (lanes 3), or with PBS (lanes 4). Immunocomplexes were detected using biotinylated rabbit anti-turkey IgG (H+L) followed by avidin conjugated to alkaline phosphatase with 4-chloro-1-naphthol as the substrate. The 97.4K, 60K, and major outer membrane protein (MOMP) are labeled on the left.


A
1 2 3 4
97.4
60
MOMP
B
1 2 3 4
97.4
60
MOMP
C
1 2 3 4
97.4
60
MOMP

DISCUSSION

Comparisons of the OMP profiles of TT3, VS1, and B577 showed distinct patterns (fundamental differences) between strains. Differences in electrophoretic patterns among the strains were noted for the major band (MOMP) in the 39,000 to 42,000 molecular weight region and several bands of relatively high molecular weights (85-105K). These differences between strains may relate to grouping chlamydia by restriction endonuclease analysis (3, 4), monoclonal specificities (5), and virulence in turkeys (16, 17, 112, 113, 114, 117, 118, 119, 149). In this study, there were also minor polypeptides of TT3, VS1, and B577 with different molecular weights. These differences in the minor polypeptide profile potentially provide further epidemiological markers and may aid in classification of chlamydial strains.

A typing strategy based on immunoreactive patterns of serum samples with a panel of OMPs from TT3, VS1, and B577 correlated with which strain of chlamydiae (TT3, VS1, or B577) was used for experimental inoculation of turkeys. The 97.4K OMP of TT3, VS1, and B577 reacted with antibodies from birds exposed to avian but not to mammalian C. psittaci. Fewer bands and often weaker reactions were detected by immunoblotting sera from TT3- or VS1-inoculated turkeys with heterologous avian antigens than with homologous antigens.

Also, these sera reacted with MOMP of B577 and the homologous strain, but not consistently with the heterologous avian strain. In contrast, sera from B577-inoculated birds weakly reacted with MOMP from each strain.

A recent study (162) has demonstrated that rabbit hyper-immune sera produced against an avian isolate from either high- or low-infectivity groups react with the MOMP of isolates from their respective groups. However, a separate study (50) has shown that rabbit hyper-immune sera produced against a mammalian strain (Feline/145) reacted with MOMPs of chlamydiae from parakeet (Prk/Daruma), muskrat (M56), and feline (Fe/Pn-1, Fe/145) strains. The MOMP of the parakeet strain (Prk/Daruma) reacts with monoclonal antibodies produced to MOMP of a parrot strain (Prt/GCP-1) which cross-react with MOMPs of avian (psittacine and non-psittacine) strains, including those of turkey strains (51). These studies indicate the necessity for further investigations involving the immunogenicity of MOMPs in the natural host.

Antibodies from TT3-inoculated birds reacted with many different OMPs of TT3, VS1, and B577. The MOMP, 60K, and 97.4K OMPs were the predominant antigens recognized by antibodies in sera from these birds. Similar patterns were detected in plasma and joint fluid from birds intramuscularly inoculated with TT3 or from birds housed with TT3-infected

birds*. Also, antibodies reacting with the 97.4K OMP were demonstrated by day 10 after experimental inoculation with TT3. Such antibodies persisted for at least 142 days post-inoculation.

Immunoblot analysis indicates that B577-inoculated turkeys failed to produce detectable antibodies to 97.4K OMP; this may have been due to the presence of independent controls at the level of transcription or translation of genes encoding this protein. The regulation of these or other processes that control biosynthesis of this OMP, or ability of the organism to multiply or persist in turkeys, is a crucial aspect of the mechanisms that may contribute to virulence in turkeys. Growth factors and/or post-translational modifications of precursors or intermediates of biosynthetic pathways of B577 may be altered or degraded in turkey (respiratory) tissues. Intracellular growth of C. psittaci is dependent on appropriate amino acid concentrations (2) and can be inhibited by gamma interferon (31).

Isolation and identification of chlamydia constitute a conclusive diagnosis, but may require two to three weeks when blind passages are needed for isolation. This is of limited value when chlamydial outbreaks in turkey flocks occur. The immunological typing described in this study should aid in

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*Jensen, A. E. National Animal Disease Center, Ames, Iowa: Unpublished data, 1988.

diagnostic evaluation of avian chlamydiosis by determining high- or low-virulent C. psittaci outbreaks in turkey flocks.

Virulent turkey strains (e.g., TT3) cause acute epornitics in which 5 to 30% of affected birds die. These strains are transmissible to humans. The disease caused by these strains is characterized by extensive vascular congestion and inflammation of vital organs. In contrast, low-virulent strains (e.g., VS1 or "psittacine-like" strains) produce progressive epornitics with less than 5% mortality when uncomplicated by secondary infection. The disease usually does not develop the severe vascular damage evident in birds infected with virulent strains. Humans are rarely infected with low-virulent strains unless unusual conditions alter the balance between infection and resistance (119).

Low-virulent strains are often isolated from pigeons, ducks, and occasionally turkeys, sparrows, and other wild birds. Pigeons are sensitive to several mildly virulent strains from pigeons and sparrows as well as some mammalian strains. However, pigeons are innocuous (excrement shedding occurs) to virulent turkey strains and to some strains from domestic herbivores (e.g., B577) (119). Therefore, pigeons and other birds not only serve as a potential reservoir but there seems to be a protective mechanism which prevents certain strains from damaging their tissues.

Recently Winsor and Grimes (162) have separated avian C. psittaci on the basis of infectivity and cytopathology in L-

929 tissue culture cells. Antigenic composition using rabbit hyper-immune sera and variations in molecular weight of the MOMPs correlated with differences in infectivity. Hyper-immune sera produced against a high- or low-infectivity group isolate reacted with the 97K and 60K OMP antigens of both high- and low-infectivity groups, but reacted only with the MOMP of isolates from their respective groups. These results strongly agree with the antibody-antigen reactions detected when using sera from turkeys experimentally exposed to avian C. psittaci but not to mammalian C. psittaci.

In turkeys, the role of the humoral immune response against C. psittaci is not clear. Antibodies in sera of turkeys exposed to avian strains reacted with heterologous as well as with homologous OMPs. This suggests that OMPs carry common antigenic epitopes among the strains. The production of specific antibodies to these determinants may play a role in defense mechanisms. The nature of protective antigens and distribution among avian C. psittaci is difficult to determine; this is complicated by difficulty in separating antigens. Therefore, erroneous results in the search for protective chlamydial antigens in turkeys may be encountered when birds other than turkeys (e.g., pigeons) or mammals are used. Antigens responsible for a protective response in turkeys may not be the same as for mammals or other birds. A homologous system would necessitate the use of immune-sera of turkeys to identify protective antigens.

Immunochemical properties of OMPs demonstrated in this study could provide information for development of a potential vaccine and may provide improved methods for diagnostic evaluation of turkeys infected with C. psittaci. The immunological typing strategy using sera from turkeys exposed to avian or mammalian C. psittaci which contain antibodies that react with antigens of specific strains of chlamydiae may prove to be a valuable method to determine high- or low-virulent C. psittaci outbreaks of turkey flocks. Differences of OMPs may influence the nature of disease caused by these pathogens. The OMPs and associated receptors that allow the organism to attach, evade, or interfere with the immune surveillance system of the host have been reported (101, 120, 136). However, the fate of chlamydiae in the host needs further investigation including the initiation and maintenance of the carrier state in turkeys.

GENERAL SUMMARY AND DISCUSSION

The humoral antibody response in turkeys experimentally exposed to Chlamydia psittaci is active in the production of specific antibodies against surface components of chlamydiae. Antibody reactivity of plasma and joint fluid samples of birds inoculated with avian C. psittaci demonstrates that 97.4K outer membrane protein (OMP), the 60K proteins, and the major OMP are the predominant antigens detected by immunoblot analysis. Immunoblot techniques show that antigens to which these antibodies react are protein in nature. In addition, antibodies which react with antigens of specific strains of chlamydia are produced in turkeys exposed to other avian and mammalian C. psittaci.

Immunoblotting techniques have demonstrated antibodies to chlamydiae membrane proteins in humans with genital infections of C. trachomatis (108), in sheep vaccinated with an ovine abortion strain (94), in monkeys experimentally inoculated with a human trachoma strain (36), and in guinea pigs which were used as a model for human genital infection using the guinea pig inclusion conjunctivitis (GPIC) strain of C. psittaci (14). In the present study, immunoblot analysis examines turkey humoral antibody response against OMPs from elementary bodies of C. psittaci during the course of experimental infection with the virulent avian (turkey) strain TT3. Immunoblot analysis of plasma from turkeys

infected with avian chlamydia detects antibodies against chlamydial antigens, particularly to the 97.4K OMP. Results of this study identifies chlamydial antigens which stimulate the immune response of turkeys during infection; these antigens may be important in the production of protective antibodies.

In Part I of this study, we used an immunoglobulin G (IgG) immunoblot assay to examine turkey humoral antibody response against OMPs from elementary bodies of C. psittaci during the course of experimental infection with the virulent avian (turkey) strain TT3. Detection of circulating antibodies against these OMPs demonstrates that the humoral immune response is involved in chlamydial infection of turkeys. Results reported herein suggest that carrier birds or birds with recently acquired chlamydial infections may be detected by immunoblot analysis of their sera with OMPs from TT3. A seronegative bird would indicate no previous contact with TT3, whereas a seropositive bird could be a potential carrier.

The major OMP, the 60K proteins, and a 97.4K OMP were the predominant antigens recognized by IgG in plasma and joint fluid from turkeys experimentally exposed to TT3. Plasma IgG specific for the 97.4K protein band was first detected at post-inoculation day (PID) 10 and was detectable to PID 142. Antibodies to the 60K and MOMP were variable and were first detected as early as PID 14-17 and PID 7-10 in some birds and

as late as PID 36-42 and PID 42-70 in others, respectively. Immunoblotting techniques showed that the antigens to which these antibodies were reacting were protein in nature.

Part II of this study investigated immunochemical properties of OMPs from two avian and one mammalian strains of C. psittaci which represent distinct C. psittaci groups as defined by restriction endonuclease analysis (3,4), monoclonal antibody specificity (5), and virulence for turkeys (149). Immunological typing based on antibody reactions with antigens of specific strains of chlamydiae using sera from turkeys exposed to avian or mammalian chlamydiae may prove to be valuable in determining C. psittaci strains of high- or low-virulence in outbreaks of chlamydiosis in turkey flocks.

Results of this study has implications for development of serodiagnostic assays as well as the identification of potential OMPs for subunit immunogens (vaccines) in birds. Differences in immunochemical properties between avian and mammalian strains are useful for the differentiation into separate biovars and may relate to their differences in pathogenicity.

REFERENCES

1. **Alexander, J. J.** 1968. Separation of protein synthesis in meningopneumonitis agent from that in L cells by differential susceptibility to cyclohexamide. J. Bacteriol. **95**:327-332.

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

3. **Andersen, A. A.** 1987. Comparisons of Chlamydia psittaci isolates from psittacine and nonpsittacine birds by DNA restriction endonuclease analysis. Abstr. no. D155, p. 97. 87th Annu. Meet. Am. Soc. Microbiol., Atlanta, Ga.

4. **Andersen, A. A., and C. A. Bolin.** 1986. Comparisons of Chlamydia psittaci isolates from turkeys with mammalian and parakeet isolates by DNA restriction endonuclease analysis. Abstr. no. D166, p. 93. 86th Annu. Meet. Am. Soc. Microbiol., Washington, D. C.

5. **Andersen, A. A., and R. A. Van Deusen.** 1988. Production and partial characterization of monoclonal antibodies to four Chlamydia psittaci isolates. Infect. Immun. **56**:2075-2079.

6. **Anderson, D. C., P. A. Stoesz, and A. F. Kaufmann.** 1978. Psittacosis outbreak in employees of a turkey-processing plant. Am. J. Epidemiol. **107**:140-148.

7. **Andral, B., C. Louzis, D. Trap, J. A. Newman, G. Bennejean, and R. Gaumont.** 1985. Respiratory disease (rhinotracheitis) in turkeys in Brittany, France, 1981-1982. I. Field observations and serology. Avian Dis. **29**:26-34.

8. **Andral, B., M. Metz, D. Toquin, J. LeCoz, and J. Newman.** 1985. Respiratory disease (rhinotracheitis) of turkeys in Brittany, France. III. Interaction of multiple infecting agents. Avian Dis. **29**:233-243.

9. **Arnstein, P., and K. F. Meyer.** 1982. Revised by J. Schachter. Psittacosis and ornithosis. p. 528-534. In M. L. Petrak (ed.), Diseases of cage and aviary birds. Lea and Febiger, Philadelphia.

10. **Bankoski, R. A., H. Gerlach, and T. Mikami.** 1968. Histologic changes and inclusion bodies in chickens inoculated with an ornithosis agent. Avian Dis. **12**:217-226.

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

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

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

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

15. **Beachey, E. H.** 1981. Bacterial adherence: Adhesive-receptor interactions mediating the attachment of bacteria to mucosal surfaces. J. Infect. Dis. **143**:325-345.

16. **Beasley, J. N., D. E. Davis, and L. C. Grumbles.** 1959. Preliminary studies on the histopathology of experimental ornithosis in turkeys. Am. J. Vet. Res. **20**:341-349.

17. **Beasley, J. N., D. E. Davis, and J. R. Watkins.** 1961. Experimental psittacosis and ornithosis in turkeys. II. Comparison of the histopathology of nine strains. Avian Dis. **5**:94-101.

18. **Becker, Y.** 1978. The chlamydiae: Molecular biology of procaryotic obligate parasites of eucaryotes. Microbiol. Rev. **42**:274-306.

19. **Bendayan, M.** 1984. Protein A-gold electron microscopic immunocytochemistry: Methods, applications, and limitations. J. Electron. Microsc. Tech. **1**:243-270.

20. **Benedict, A. A., and E. O'Brien.** 1956. Antigenic studies on the psittacosis-lymphogranuloma venereum group of viruses. II. Characterization of complement fixing antigens extracted from sodium lauryl sulfate. J. Immunol. **76**:293-300.

21. **Bevan, B. J., J. Labrain.** 1983. Laboratory transfer of a virus between isolates of Chlamydia psittaci. Vet. Rec. **112**:280-285.

22. **Birkelund, S., A. G. Lundemose, and G. Christiansen.** 1988. Chemical cross-linking of Chlamydia trachomatis. Infect. Immun. **56**:654-659.

23. **Bovarnick, M. R., J. C. Miller, and J. C. Snyder.** 1950. The influence of certain salts, amino acids, sugars, and proteins on the stability of rickettsiae. J. Bacteriol. **59**:509-522.

24. **Bracewell, C. D., and B. J. Bevan.** 1986. Chlamydiosis in birds in Great Britain 1. Serological reactions to chlamydia in birds sampled between 1974 and 1983. J. Hyg. Camb. **96**:447-451.

25. **Brade, L., S. Schramek, U. Schade, and H. Brade.** 1986. Chemical, biological, and immunochemical properties of the Chlamydia psittaci lipopolysaccharide. Infect. Immun. **54**:568-574.

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

27. **Brenner, S., and R. W. Horne.** 1959. A negative-staining method for high resolution electron microscopy of viruses. Biochim. Biophys. Acta **34**:103-110.

28. **Brown, M. R. W., and P. Williams.** 1985. The influence of environment on envelope properties affecting survival of bacteria in infection. Annu. Rev. Microbiol. **39**:527-556.

29. **Brubaker, R. R.** 1985. Mechanisms of bacterial virulence. Annu. Rev. Microbiol. 39:21-50.

30. **Buzoni-Gatel, D., K. Layachi, G. Dubray, and A. Rodolakis.** 1989. Comparison of protein patterns between invasive and non-invasive ovine strains of Chlamydia psittaci. Res. Vet. Sci. **46**:40-42.

31. **Byrne, G. I., L. K. Lehmann, and G. J. Landry.** 1986. Induction of tryptophan catabolism is the mechanism for gamma-interferon-mediated inhibition of intracellular Chlamydia psittaci replication in T24 cells. Infect. Immun. **53**:347-351.

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

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

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

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

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

37. **Campbell, R. S. F.** 1986. The pathogenesis and pathology of avian respiratory infections. Vet. Bull. **56**:521-543.

38. **Chernesky, M. A., J. B. Mahony, S. Castriciano, M. Mores, I. O. Stewart, S. J. Landis, W. Seidelman, E. J. Sargeant, and C. Leman.** 1986. Detection of Chlamydia trachomatis antigens by enzyme immunoassay and immunofluorescence in genital specimens from symptomatic and asymptomatic men and women. J. Infect. Dis. **154**:141-148.

39. **Cox, R. L., C. -C. Kuo, J. T. Grayston, and L. A. Campbell.** 1988. Deoxyribonucleic acid relatedness of Chlamydia sp. strain TWAR to Chamydia trachomatis and Chlamydia psittaci. Int. J. Syst. Bacteriol. **38**:265-268.

40. **Davis, D. E., J. R. Delaplane, and J. R. Watkins.** 1957. The role of turkey eggs in the transmission of ornithosis. Am. J. Vet Res. 18:409-413.

41. **DeMey, J.** 1984. Collodial gold as marker and tracer in light and electron microscopy. EMSA **14:** 54-66.

42. **Dhingra, P. N., L. P. Agarwal, V. M. Mahajan, S. C. Adlakha, and K. K. Baxi.** 1981. Chlamydial group antigen: Its preparation and use in complement fixation test for measuring chlamydial group antibodies in animals. Zbl. Vet. Med. B, 28:336-340.

43. **Dominick, M. A., M. J. F. Schmerr, and A. E. Jensen.** 1985. Expression of type 1 pili by Escherichia coli strains of high and low virulence in the intestinal tract of gnotobiotic turkeys. Am. J. Vet. Res. **46:** 270-275.

44. **Durfee, P. T., M. M. Pullen, R. W. Currier Jr., and R. L. Parker.** 1975. Human psittacosis associated with commercial processing of turkeys. J. Am. Vet. Med. Assoc. **167:**804-808.

45. **Eddie, B., K. F. Meyer, F. L. Lambrecht, and D. P. Furman.** 1962. Isolation of ornithosis bedsoniae from mites collected in turkey quarters and from chicken lice. J. Infect. Dis. **110:**231-237.

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

47. **Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of Escherichia coli by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. **115:**717-722.

48. **Frere, J.-M., and B. Joris.** 1985. Penicillin-sensitive enzymes in peptidoglycan biosynthesis. CRC Crit. Rev. Microbiol. **11:**229-238.

49. **Friis, R. R.** 1972. Interaction of L cells and Chlamydia psittaci: Entry of the parasite and host responses to its development. J. Bacteriol. **180:**706-721.

50. **Fukushi, H., and K. Hirai.** 1988. Immunochemical diversity of the major outer membrane protein of avian and mammalian Chlamydia psittaci. J. Clin. Microbiol. **26**:675-680.

51. **Fukushi, H., K. Nojiri, and K. Hirai.** 1987. Monoclonal antibody typing of Chlamydia psittaci strains derived from avian and mammalian species. J. Clin. Microbiol. **25**:1978-1981.

52. **Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond, and M. J. Warring.** 1981. The molecular basis of antibiotic action. John Wiley & Sons, New York.

53. **Garrett, A. J., M. J. Harrison, and G. P. Manire.** 1974. A search for the bacterial mucopeptide component muramic acid in Chlamydia. J. Gen. Microbiol. **80**:315-318.

54. **Grayston, J. T., C. -C. Kuo, L. A. Campbell, and S. -P. Wang.** 1989. "Chlamydia-pneumoniae" new species for Chlamydia-sp. strain TWAR. Int. J. Syst. Bacteriol. **39**:88-90.

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

56. **Grimes, J. E.** 1978. Transmission of chlamydiae from grackles to turkeys. Avian Dis. 22:308-312.

57. **Grimes, J. E.** 1984. Serological and microbiological detection of Chlamydia psittaci infections in psittacine birds. Avian/Exotic Pract. 1:6-12.

58. **Grimes, J. E.** 1985. Enigmatic psittacine chlamydiosis: results of serotesting and isolation attempts, 1978 through 1983, and consideration for the future. J. Am. Vet. Med. Assoc. **186**:1075-1079.

59. **Grimes, J.** 1986. Chlamydia psittaci latex agglutination antigen[A] for rapid detection of antibody activity in avian sera: Comparison with direct complement fixation and isolation results. Avian Dis. **30**:60-66.

60. **Grimes, J. E., K. J. Owens, and J. R. Singer.** 1979. Experimental transmission of Chlamydia psittaci to turkeys from wild birds. Avian Dis. 23:915-926.

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

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

63. **Hall, C. F., S. E. Glass, J. E. Grimes, and R. W. Moore.** 1975. An epidemic of ornithosis in Texas turkeys in 1974. Southwest Vet. **28**:19-21.

64. **Hancock, K., and V. C. W. Tsang.** 1983. India ink staining of proteins on nitrocellulose paper. Anal. Biochem. **133**:157-162.

65. **Hanna, L., T. C. Merigan, and E. Jawetz.** 1966. Inhibition of TRIC agents by virus-induced interferon. Proc. Soc. Exp. Biol. Med. **122**:417-421.

66. **Hanna, L., T. C. Merigan, and E. Jawetz.** 1967. Effect of interferon on TRIC agents and induction of interferon by TRIC agents. Am. J. Ophthal. **63**:1115/89-1119/93.

67. **Hatch, T. P.** 1988. Metabolism of chlamydia. p. 97-109. In A. L. Barron (ed.), Microbiology of chlamydia. CRC Press, Inc., Boca Raton, Florida.

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

69. **Hatch, T. P., M. Miceli, and J. E. Sublett.** 1986. Synthesis of disulfide-bonded outer membrane proteins during the developmental cycle of Chlamydia psittaci and Chlamydia trachomatis. J. Bacteriol. **165**:379-385.

70. **Hatch, T. P., D. W. Vance, Jr., and E. Al-Hossainy.** 1981. Identification of a major envelope protein in Chlamydia spp. Infect. Immun. **146**:426-429.

71. **Hayat, M. A.** 1981. Fixation for electron microscopy. Academic Press, Inc., New York.

72. **Higashi, N.** 1965. Electron microscopic studies on the mode of reproduction of trachoma virus and psittacosis virus in cell culture. Exp. Mol. Pathol. **4**:24-39.

73. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among Salmonella lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154**:269-277.

74. **Horn, J. E., E. W. Kappus, S. Falkow, and T. C. Quinn.** 1988. Diagnosis of Chlamydia trachomatis in biopsied tissue specimens by using in situ DNA hybridization. J. Infect. Dis. **175**:1249-1252.

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

76. **Howard, L., N. S. Orenstein, and N. W. King.** 1974. Purification of renografin density gradients of Chlamydia trachomatis grown in the yolk sac of eggs. Appl. Microbiol. **27**:102-106.

77. **Hyypia, T., A. Jalava, S. H. Larsen, P. Terho, and V. Hukkanen.** 1985. Detection of Chlamydia trachomatis in clinical specimens by nucleic acid spot hybridization. J. Gen. Microbiol. **131**:975-978.

78. **Irons, J. V., T. D. Sullivan, and J. Rowen.** 1951. Outbreak of psittacosis (ornithosis) from working with turkeys or chickens. Am. J. Public Health **41**:931-937.

79. **Isaacson, R. E.** 1985. Pilus adhesins. p. 307-336. In D. C. Salvage, and M. Fletcher (ed.), Bacterial adhesion mechanisms and physiological significance. Plenum Press, New York, New York.

80. **Jenkin, H. M., and Y. K. Lu.** 1967. Induction of interferon by the BOUR strain of trachoma on HeLa 229 cells. Am. J. Ophthalmol. **63**:1110/84-1115/89.

81. **Jenkin, H. M., M. R. Ross, and J. W. Moulder.** 1961. Species-specific antigens from the cell walls of the agents of meningopneumonitis and feline pneumonitis. J. Immunol. **86**:123-127.

82. **Jones, R. B., S. C. Bruins, W. J. Newhall.** 1983. Comparison of reticulate and elementary body antigens in detection of antibodies against Chlamydia trachomatis by an enzyme-linked immunosorbent assay. J. Clin. Microbiol. **17**:466-471.

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

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

85. **Lewis, V. J., W. L. Thacker, and S. H. Mitchell.** 1977. Enzyme-linked immunosorbent assay for chlamydial antibodies. J. Clin. Microbiol. **6**:507-510.

86. **Lin, H. -S., and J. W. Moulder.** 1966. Patterns of response to sulfadiazine, D-cycloserine, and D-alanine in members of the psittacosis group. J. Infect. Dis. **116**:372-376.

87. **Lipkin, E. S., J. V. Moncada, M. Shafer, T. E. Wilson, and J. E. Schachter.** 1986. Comparison of monoclonal antibody staining and culture in diagnosing cervical chlamydiae infection. J. Clin. Microbiol. **23**:114-117.

88. **Maeland, J. A.** 1968. Antigenic properties of various preparations of Neisseria gonorrhoeae endotoxin. Acta Pathol. Microbiol. Scandinav. **73**:413-422.

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

90. **Matikainen, M. -T., and P. Terho.** 1983. Immunochemical analysis of antigenic determinants of Chlamydia trachomatis by monoclonal antibodies. J. Gen. Microbiol. **129**:2343-2350.

91. **Matsumoto, A., N. Higashi, and A Tamura.** 1973. Electron microscopic observations on the effect of polymyxin B on cell walls of Chlamydia psittaci. J. Bacteriol. **113**:357-364.

92. **Matsumoto, A., and G. P. Manire.** 1970. Electron microscopic observations on the effects of penicillin on the morphology of Chlamydia psittaci. J. Bacteriol. **101**:278-285.

93. **McClenaghan, M., A. J. Herring, and I. D. Aitken.** 1984. Comparison of Chlamydia psittaci isolates by DNA restriction endonuclease analysis. Infect. Immun. **45**:384-389.

94. **McClenaghan, M., A. J. Herring, I. D. Aitken, and J. R. Honeycombe.** 1986. Some comparative biochemical studies on Chlamydia psittaci strains of ovine and avian origin. p. 139-148. In I. D. Aitken (ed.), Agriculture chlamydial diseases of ruminants. Commission of the European Communities, Luxembourg.

95. **Mearns, G., S. J. Richmond, and C. C. Storey.** 1988. Sensitive immune dot blot test for diagnosis of Chlamydia trachomatis infection. J. Clin. Microbiol. **26**:1810-1813.

96. **Menozzi, F. D., C. Menozzi-Dejaiffe, and F. E. Nano.** 1989. Molecular cloning of a gene encoding a Chlamydia psittaci 57-kDa protein that shares antigenic determinants with ca. 60-kDa proteins present in many gram-negative bacteria. FEMS Microbiol. Lett. **58**:59-64.

97. **Meyer, K. F.** 1967. The host spectrum of psittacosis-lymphogranuloma venereum (PL) agents. Am. J. Ophthalmol. **63**:1225-1245.

98. **Moore, F. M., and M. L. Petrak.** 1985. Chlamydia immunoreactivity in birds with psittacosis: Localization of chlamydiae by the peroxidase-antiperoxidase method. Avian Dis. **29**:1036-1042.

99. **Morrison, R. P., K. Lyng, and H. D. Caldwell.** 1989. Chlamydial disease pathogenesis, ocular hypersensitivity elicited by a genus-specific 57-kD protein. J. Exp. Med. **169**:663-675.

100. **Moulder, J. W.** 1962. Structure and chemical composition of isolated particles. Ann. N. Y. Acad. Sci. **98**:92-99.

101. **Moulder, J. W.** 1985. Comparative biology of intracell. Microbiol. Rev. **49**:298-337.

102. **Moulder, J. W., T. P. Hatch, C. -C. Kuo, J. Schachter, and J. Storz.** 1984. Genus I. Chlamydia. p. 729-739. In N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology. vol. 1. The Williams & Wilkins Co., Baltimore.

103. **Moulder, J. W., N. J. Levy, and L. P. Schulman.** 1980. Persistent infection of mouse fibroblasts (L cells) with Chlamydia psittaci: Evidence for a cryptic chlamydial form. Infect. Immun. **30**:874-883.

104. **Moulder, J. W., D. L. Novasel, and J. E. Officer.** 1963. Inhibition of the growth agents of the psittacosis group by D-cycloserine and its specific reversal by D-alanine. J. Bacteriol. **85**:707-711.

105. **Narita, T., P. B. Wyrick, and G. P. Manire.** 1976. Effects of alkali on the structure of cell envelopes of Chlamydia psittaci elementary bodies. J. Bacteriol. **125**:300-307.

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

107. **Newhall, W. J., V.** 1988. Macromolecules and antigenic composition of chlamydiae. p. 47-70. In A. L. Barron (ed.), Microbiology of chlamydia. CRC Press, Inc., Boca Raton, Florida.

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

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

110. **Newhall, W. J., V, P. Terho, C. E. Wilde III, B. E. Batteiger, and R. B. Jones.** 1986. Serovar determination of Chlamydia trachomatis isolates by using type-specific monoclonal antibodies. J. Clin. Microbiol. **23**:333-338.

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

112. **Page, L. A.** 1959. Experimental ornithosis in turkeys. Avian Dis. **3**:51-66.

113. **Page, L. A.** 1966. Interspecies transfer of psittacosis-LGV-trachoma agents: Pathogenicity of two avian and two mammalian strains for eight species of birds and mammals. Am. J. Vet. Res. **27**:397-407.

114. **Page, L. A.** 1967. Comparisons of "pathotypes" among chlamydial (psittacosis) strains recovered from diseased birds and mammals. Bull. Wildlife Dis. Assoc. **3**:166-175.

115. **Page, L. A.** 1974. Application of an agar gel precipitin tests to the serodiagnosis of avian chlamydiosis. Am. Assoc. Vet. Lab. Diag. **17**:51-61.

116. **Page, L. A.** 1975. Studies on immunity to chlamydiosis in birds, with particular reference to turkeys. Am. J. Vet. Res. **36**:597-600.

117. **Page, L. A., and R. A. Bankowski.** 1959. Investigation of a recent ornithosis epornitic in California Turkeys. Am. J. Vet. Res. **20**:941-945.

118. **Page, L. A., W. T. Derieux, and R. C. Cutlip.** 1975. An epornitic of fatal chlamydiosis (ornithosis) in South Carolina turkeys. Am. Vet. Med. J. **166**:175-178.

119. **Page, L. A., and J. E. Grimes.** 1984. Avian chlamydiosis (ornithosis). p. 283-308. In M. S. Hofstad (ed.), Diseases of poultry. Iowa State University, Ames, Iowa.

120. **Pearce, J. H.** 1986. Early events in chlamydial infections. Annu. Inst. Pasteur/Microbiol. **137**:325-332.

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

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

123. **Reynolds, E. S.** 1963. Lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17**:208.

124. **Rice, C. E.** 1948. Inhibitory effects of certain avian and mammalian antisera in specific complement-fixation systems. J. Immunol. **59**:365-378.

125. **Richmond, S. J., P. Stirling, and C. R. Ashley.** 1982. Virus infecting the reticulate bodies of an avian strain of Chlamydia psittaci. FEMS Microbiol. Lett. **14**:31-33.

126. **Roberts, J. P., and J. E. Grimes.** 1978. Chlamydia shedding by four species of wild birds. Avian Dis. **22**:698-706.

127. **Robinson, E. N., Jr., Z. A. McGee, and C. M. Clemens.** 1987. Probing the surface of bacteria: Use of gold sphere immunological probes. Microbiol. Pathogenesis **2**:159-169.

128. **Rodolakis, A., F. Bernard, A. Souriau, K. Layachi, and D. Buzoni-Gatel.** 1989. Relationship between virulence of Chlamydia psittaci strains and establishment of persistent infection of McCoy cells. Vet. Microbiol. **19**:65-73.

129. **Ruppanner, R., D. E. Behymer, W. J. DeLong III, C. E. Franti, and T. Schulz.** 1984. Enzyme immunoassay of chlamydia in birds. Avian Dis. 28:608-615.

130. **Saikku, P., S. P. Wang, M. Kleemola, E. Brander, E. Rusanen, and J. T. Grayston.** 1985. An epidemic of mild pneumonia due to an unusual strain of Chlamydia psittaci. J. Infect. Dis. **151**:832-839.

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

132. **Sardinia, L. M., E. Segal, and D. Ganem.** 1988. Developmental regulation of the cysteine-rich outer membrane proteins of murine Chlamydia trachomatis. J. Gen. Microbiol. **134**:997-1004.

133. **Schachter, J.** 1978. Chlamydial infections. N. Engl. J. Med. **298**:428-435, 490-495, 540-549.

134. **Schachter, J., J. Banks, N. Sugg, M. Sung, J. Storz, and K. F. Meyer.** 1974. Serotyping of Chlamydia. I. Isolates of ovine origin. Infect. Immun. **9**:92-94.

135. **Schachter, J., J. Banks, N. Sugg, M. Sung, J. Storz, and K. F. Meyer.** 1975. Serotyping of Chlamydia: Isolates of bovine origin. Infect. Immun. **11**:904-907.

136. **Schachter, J., and H. D. Caldwell.** 1980. Chlamydiae. Ann. Rev. Microbiol. **34**:285-309.

137. **Schmeer, N.** 1983. Enzymimmuntest zum nachweis von IgG- und IgM- Antikorpern gegen Chlamydia psittaci bei der taube. Zbl. Vet. Med. B **30**:356-370.

138. **Sears, S. D., K. Richardson, C. Young, C. D. Parker, and M. M. Levine.** 1984. Evaluation of the human immune response to outer membrane proteins of Vibrio cholerae. Infect. Immun. **44**:439-444.

139. **Seki, C., I. Takashima, J. Arikawa, and N. Hashimoto.** 1988. Monoclonal antibodies to Chlamydia psittaci: Characteristics and antigenic analysis. Jpn. J. Vet. Sci. **50**:383-393.

140. **Stempak, J. G., and R. T. Ward.** 1964. An improved staining method for electron microscopy. J. Cell Biol. **22**:697-701.

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

142. **Storz, J.** 1966. Psittacosis-lymphogranuloma infection of sheep. Antigenic structures and interrelations of PL agents associated with polyarthritis, enzootic abortion, intrauterine and latent intestinal infections. J. Comp. Pathol. **76**:351-362.

143. **Storz, J.** 1971. Chlamydia and Chlamydia-induced diseases. Charles C. Thomas, Publ., Springfield, Illinois.

144. **Stryer, L.** 1981. Biochemistry. W. H. Freeman and Company, New York.

145. **Takahashi, T., I. Takashima, and N. Hashimoto.** 1988. A chicken model of systemic infection with Chlamydia psittaci: Comparison of the virulence among avian and mammalian strains. Jpn. J. Vet. Sci. **50**:622-631.

146. **Tamura, A., and N. Higashi.** 1967. Purification and chemical composition of meningopneumonitis virus. Virology **20**:596-604.

147. **Tamura, A., A. Matsumoto, and N. Higashi.** 1967. Purification and chemical composition of reticulate bodies of the meningopneumonitis organisms. J. Bacteriol. **93**:2003-2008.

148. **Tamura, A., A. Matsumoto, G. P. Manire, and N. Higashi.** 1971. Electron microscopic observations on the structure of envelopes of mature elementary bodies and development reticulate forms of Chlamydia psittaci. J. Bacteriol. **105**:355-360.

149. **Tappe, J. P., A. A. Andersen, N. F. Cheville.** 1987. Respiratory and pericardial pathology of turkeys infected with avian or mammalian strains of Chlamydia psittaci. Vet. Pathol. (accepted for publication).

150. **Timms, P., F. W. Eaves, B. J. Rodwell, and M. F. Lavin.** 1988. Comparison of DNA-spot hybridization, cell culture and direct immunofluorescence staining for the diagnosis of avian chlamydiae. Vet. Microbiol. **18**:15-25.

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

152. **Treharne, J. D., T. Forsey, and B. J. Thomas.** 1983. Chlamydial serology. Br. Med. Bull. **39**:194-200.

153. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119**:115-119.

154. **Wagar, E. A., and R. S. Stephens.** 1988. Developmental-form-specific DNA-binding proteins in Chlamydia spp. Infect. Immun. 56:1678-1684.

155. **Wang, S. -P., and J. T. Grayston.** 1970. Immunologic relationship between genital TRIC, lymphogranuloma venereum, and related organisms in a new microtiter indirect immunofluorescence test. Am. J. Ophthalmol. **70**:367-375.

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

157. **Waterlow, J. C., P. J. Garlick, and D. J. Millward.** 1978. The mechanism of protein degradation: Protein turnover in mammalian tissues and in the whole body. North-Holland Publishing Co., New York.

158. **Weiss, E.** 1965. Adenosine triphosphate and other requirements for the utilization of glucose by agents of the psittacosis-trachoma group. J. Bacteriol. **90**:243-253.

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

160. **Weiss, E., and N. N. Wilson.** 1969. Role of exogenous adenosine triphosphate in catabolic and synthetic activities of Chlamydia psittaci. J. Bacteriol. **97**:719-724.

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

162. **Winsor, D. K., Jr., and J. E. Grimes.** 1988. Relationship between infectivity and cytopathology for L-929 cells, membrane proteins, and antigenicity of avian isolates of Chlamydia psittaci. Avian Dis. **32**:421-431.

163. **Woolcock, J. B.** 1985. Pathogenesis of bacterial infections: Some determinants of virulence in gram-negative bacteria. Austr. Vet. J. **62**:177-181.

164. **Wyrick, P. B., and E. A. Brownridge.** 1978. Growth of Chlamydia psittaci in macrophages. Infect. Immun. **19**:1054-1060.

165. **Wyrick, P. B., E. A. Brownridge, and B. E. Ivins.** 1978. Interaction of Chlamydia psittaci with mouse peritoneal macrophages. Infect. Immun. **19**:1061-1067.

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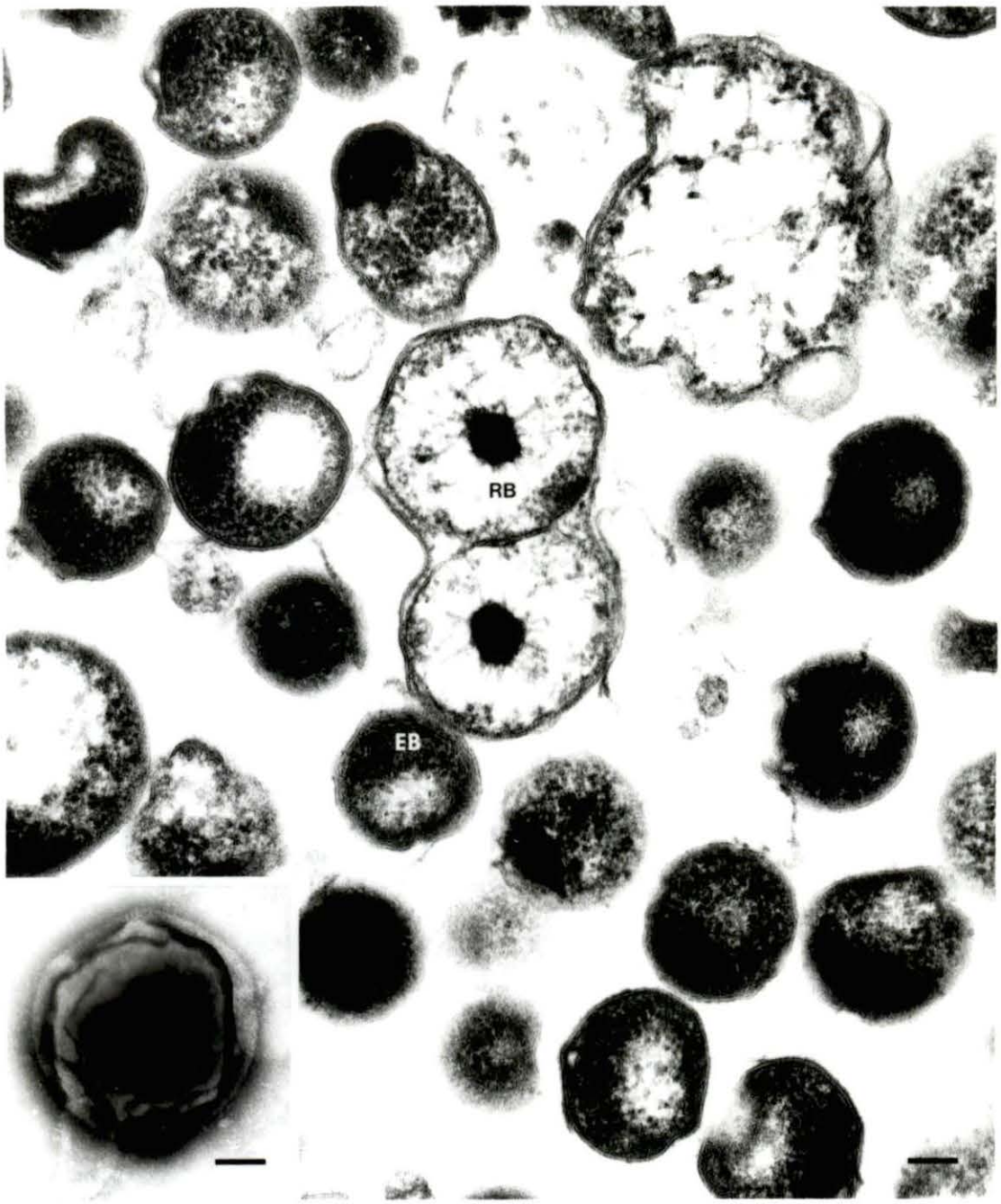
APPENDIX: ELECTRON MICROSCOPY OF *CHLAMYDIA PSITTACI* TT3

Electron micrograph showing elementary bodies (EB) and reticulate bodies (RB) of Chlamydia psittaci TT3 released from Vero cells at 48 h post-infection. Insert, elementary body stained with phosphotungstic acid. Bars, 0.1 um.


RB
EB