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An Oligomer of the Major Outer Membrane Protein of *Chlamydia psittaci* Is Recognized by Monoclonal Antibodies Which Protect Mice from Abortion

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Monoclonal antibodies (MAbs) were generated against an ovine abortive strain of *Chlamydia psittaci*. A plaque reduction assay was used to select 19 neutralizing antibodies which appeared to be heterogeneous in isotype, specificity, and recognized proteins. Different neutralizing MAbs were tested for their protective abilities against abortion in a pregnant-mouse model. All of the protective MAbs selected had the same isotype, were serotype 1 specific, and recognized a protein of about 110 kDa by immunoblotting. The recognized epitopes were resistant to sodium dodecyl sulfate and reducing agents, but all of them were heat sensitive. The protein was able to form disulfide-linked polymers. Immunological cross-reaction studies with rabbit sera showed a link between the 110-kDa protein and the major outer membrane protein (MOMP). The 110-kDa protein was purified by immunoaffinity and shown to be dissociated after heating into MOMP by silver staining and immunoblotting. These results show homogeneity among protective MAbs directed to heat-sensitive epitopes located on an oligomer of the MOMP of *C. psittaci*.

Chlamydiae are obligate intracellular gram-negative bacteria which are pathogenic for humans and animals (25). The genus *Chlamydia* consists of three well-known species, *C. trachomatis*, *C. pneumoniae*, and *C. psittaci*, and a species more recently described, *C. pecorum* (12). One of the most common pathological effects induced by *C. psittaci* infection in pregnant ewes is abortion, which results from colonization of the placenta by bacteria. Ovine abortion causes significant economic losses for farmers; moreover, the observation of infections in pregnant women (7) has focused attention on *C. psittaci*. This disease can be controlled by the use of an attenuated live vaccine (22); however, the use of a recombinant protective antigen as the vaccine would be safer. Attempts have been made to develop a recombinant vaccine to prevent human infection with *C. trachomatis*. Most of these studies have focused on the major outer membrane protein (MOMP), which is the major variable surface protein of chlamydiae and is a promising candidate antigen for a vaccine (13, 36). Although partial protection against *C. psittaci* has been observed with crude MOMP preparations (3, 30), the vaccination of ewes with recombinant linear epitopes of *C. psittaci* MOMP has been unsuccessful. Immunity to *C. psittaci* has been shown to be regulated by both cellular and humoral mechanisms (9); chlamydiae can be neutralized by serum antibodies during the bacteremia which precedes placental colonization (29). Therefore, protective antigens could potentially be detected with monoclonal antibodies (MAbs). The use of an in vitro neutralization assay and a pregnant-mouse model for passive transfer of immunity has enabled the selection of neutralizing (1) and protective antibodies (8, 23). In this study we used these two methods to select protective MAbs against an abortion strain of *C. psittaci* and have demonstrated that they recognize a presumably conformational epitope on a protein of about 110 kDa. This protein was purified and shown to be a polymer of the MOMP of *C. psittaci.*

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MATERIALS AND METHODS

Chlamydial strain, culture, and purification. The AB7 ovine abortion strain of *C. psittaci* (27) was used. Bacteria were propagated in the yolk sac of an embryonated chicken and stored at −70°C before purification. Chlamydiae were purified by the method of Caldwell et al. (10) and stored at −20°C.

MAb production. BALB/c mice were immunized by intraperitoneal inoculation with live chlamydiae (10^6 PFU/ml) twice at an interval of 30 days. Spleen cells were recovered 3 days after the last inoculation. Fusion, screening, and immunoglobulin isotype determination were performed as previously described (28). The microimmunofluorescence (MIF) assay of Wang and Grayston (34) was used for screening and for the determination of MAb specificity.

Evaluation of ascitic antibody concentrations. Concentrations were measured by a sandwich enzyme-linked immunosorbent assay. Microtiter wells were coated with a goat anti-mouse antibody, dilutions of ascitic fluids were added to the wells, and a goat anti-mouse alkaline-phosphatase conjugate was used in the last step. A protein A-purified immunoglobulin G2a (IgG2a) antibody was used as the standard. All immunoglobulin concentrations ranged between 7 and 11 mg/ml.

In vitro neutralization assays. Neutralization assays were done by the plaque reduction assay of Schachter et al. (24) with McCoy cells. Dilutions of ascitic fluids in calcium- and magnesium-free phosphate-buffered saline (PBS; pH 7.3)–10% fetal bovine serum–2.5% guinea pig serum were added to an equal volume of an AB7 chlamydial suspension (2×10^6, 2×10^5, and 2×10^4 PFU/ml), incubated for 2 h at 37°C, and then titrated on McCoy cell monolayers by plaque assay. The neutralizing titer was expressed as the highest dilution which gave a 50% or greater decrease in PFU.

Pregnant-mice protection assays. Pregnant mice were passively immunized with 0.1 ml of 10^{-1} dilutions of ascitic MAbs in PBS on day 11 of gestation and intravenously challenged the following day with 2×10^5 PFU of strain AB7. Two controls, 34 mice which were infected but not immunized (virulence control group) and 25 mice neither infected nor immunized (normal control group), were used. Protection was estimated by comparing the number of living offspring per litter at 1 week postparturition by analysis of variance (F test). Mice were considered to be protected when they differed from the virulence control group ($P \leq 0.0050$).

Solubilization of chlamydial proteins. Proteins of purified bacteria were solubilized in a buffer (0.1 M NaCl, 2 mM EDTA, 50 mM Tris HCl [pH 8.8]) with 0.4% sodium dodecyl sulfate (SDS)–2.5% β-mercaptoethanol for 30 min at room temperature.

SDS-PAGE, silver staining, and immunoblot analysis. Polyacrylamide gel electrophoresis (PAGE) was performed by the Laemmli (16) procedure, except for the sample buffer, which was adjusted to pH 8.5 to facilitate disulfide bond reduction without heating. Both unheated and heated samples (100°C for 7 min) were analyzed. The immunoblot analysis was previously described (31), and blots

were reacted with a biotinylated anti-mouse whole IgG and a streptavidin-alkaline-phosphatase conjugate (Amersham Co., Amersham, United Kingdom). Silver staining was performed as previously described (15). Molecular weight markers (MW-SDS-200 kit) were obtained from Sigma Chemical Co., St. Louis, Mo.

Purification of MAbs. MAbs from ascitic fluids were precipitated with 40% ammonium sulfate and then purified on a Sepharose-protein A column (Pharmacia, Uppsala, Sweden) according to the manufacturer's recommendations.

Antibody coupling protocol. Purified antibodies were coupled to 1,1′-carbonyldiimidazole-activated 6% cross-linked beaded agarose (Reacti-Gel 6X CDI; Pierce, Rockford, Ill.) at the rate of 2 mg of antibodies per ml of gel according to the instructions of the manufacturer.

Purification of the 110-kDa protein. Proteins solubilized in SDS were diluted 10-fold in 0.3 M NaCl–50 mM iodoacetamide–50 mM Tris HCl (pH 7.4) with 0.1% Triton X-100 to reduce the SDS concentration to 0.04%. The solution was prefiltered through a 0.45-μm-pore-size filter and then centrifuged at 4,000 × *g* for 45 min on a microseparator ultrafilter with a 30-kDa cutoff (Centricon-30; Amicon, Danvers, Mass.) in order to remove SDS and β-mercaptoethanol. Samples were subjected to two additional ultra filtration cycles. The first cycle used a buffer of 0.3 M NaCl–50 mM Tris (pH 7.4), and the second used a buffer of 0.1 M NaCl–50 mM Tris (pH 7.4) with 0.1% Triton X-100. Proteins with a molecular mass of greater than 30 kDa were recovered and incubated for 1 h at room temperature with gel affinity beads. After the beads were washed with Tris–NaCl–Triton X-100 buffer, purified proteins were eluted with a buffer of 3 M $MgCl_2$–25% ethylene glycol–0.075 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 8) (33) and desalted by ultrafiltration.

N-terminal amino acid sequencing. Purified and desalted protein (3 μg) was added to the polyvinylidene difluoride membrane disk of a sample preparation cartridge (ProSpin; Applied Biosystems, Foster City, Calif.). The sample was fixed and washed with highly purified water containing dithiothreitol (2 mg/ml) by several centrifugation cycles. The polyvinylidene difluoride membrane was then removed from the cartridge and used for amino acid analysis in a Porton LF 3000 protein sequencer (Beckman Instruments, Inc., Fullerton, Calif.). Ten sequencing cycles were performed.

Production of polyclonal monospecific rabbit sera. Chlamydial proteins were solubilized, diluted with Laemmli buffer, heated to obtain MOMP or left unheated to retain the 110-kDa protein, separated by preparative SDS-PAGE, and then transferred to nitrocellulose sheets (Protran BA83 [0.2-μm pore size]; Schleicher and Schuell, Dassel, Germany). Lateral strips of the membrane were cut and reacted with MAbs to locate the protein positions. The portions of nitrocellulose (average, 2.5 cm^2) containing the proteins of interest were cut and ground in PBS. The suspensions obtained were injected subcutaneously into New Zealand White specific-pathogen-free rabbits. For each protein, two rabbits were immunized with four injections given at 15-day intervals.

RESULTS

Neutralization assays. The abilities of 28 MAbs to neutralize the infectivity of the virulent AB7 strain of *C. psittaci* in cell culture were assessed by the plaque reduction test. MAbs included the following groups: 3 genus-specific MAbs, 18 serotype 1-specific MAbs, and 7 subspecies-specific MAbs. Nineteen MAbs (Table 1) (2 genus-specific, 4 subspecies-specific, and 13 serotype 1-specific MAbs) had neutralizing effects. The molecular masses of the molecules recognized by MAbs on immunoblots are given in Table 1. Four MAbs did not react on the Western blot (immunoblot).

Pregnant-mouse protection assays. Protection assays (Fig. 1) were performed with 12 of the neutralizing MAbs (1 genus-, 2 subspecies-, and 9 serotype 1-specific MAbs). The average litter size for the 25 normal control mice was 10.8 at 1 week. For the 34 virulence control mice, the average litter size at 1 week was 0.47; 31 of these mice aborted. The numbers of live newborn mice were significantly higher ($P < 0.0001$) for groups receiving the nine IgG2a serotype 1-specific MAbs, but three of them (CA5G11, FB5B2, and JC3A5) had significantly ($P < 0.0018$, $P < 0.0139$, and $P < 0.0177$, respectively) fewer live newborn mice than did the normal control group. The three MAbs listed above were less efficacious than the others (AD4A4, EA6H5, FA2H10, GD3G5, JC6H3, and LC6D12) under the conditions tested. No genus- or subspecies-specific MAb was protective.

Western blot characterizations of MAbs. None of the nine protective MAbs gave a response by Western blot analysis after SDS-PAGE by the Laemmli procedure, with or without a reducing agent. Under reducing conditions but without heating, a protein band in the 110-kDa region was detected (Fig. 2A, lane 2) with all of the protective MAbs. A smear above the 110-kDa protein could be observed. The same results were obtained with chlamydiae grown on McCoy cells. We observed that the presence of a reducing agent is necessary to allow the migration of this protein on a 10% acrylamide gel (Fig. 2B). Without the reducing agent, MAbs reacted with a polymer which migrated at the limit between the stacking gel (4% acrylamide) and the resolving gel (10% acrylamide). An increase in the percentage of the reducing agent allowed the progressive entry of this protein on the gel.

TABLE 1. Neutralizing and protective MAbs

Specificity and MAb	Isotype	Molecular mass (kDa)[a]	MIF titer[b]	Neutralization	
				Titer[b]	Maximum (%)
Genus					
AD5A8	IgG1	<10	−5	−5	63
LC1B11	IgG1	<10	−4	−3	65
Subspecies					
BA6H6	IgG2a	30	−4	−3	50
CB3C8	IgG2a	—[c]	−4	−2	65
KDE3E4	IgG2a	—	−5	−2	50
LA2G10	IgG2a	28	−4	−4	86
Serotype 1					
AD4A4	IgG2a	110	−5	−4	82
CA5G11	IgG2a	110	−4	−4	70
DA6D6	IgG2a	—	−4	−4	66
EA4D2	IgG3	—	−3	−2	50
EA6H5	IgG2a	110	−5	−4	71
FA2H10	IgG2a	110	−5	−5	90
FA5C5	IgG2a	110	−5	−4	86
FB5B2	IgG2a	110	−5	−4	70
GD3G5	IgG2a	110	−5	−5	72
HC3E6	IgG2a	110	−5	−5	84
JC3A5	IgG2a	110	−5	−5	85
JC6H3	IgG2a	110	−5	−5	80
LC6D12	IgG2a	110	−5	−5	84

[a] Apparent molecular mass of the recognized chlamydial antigen.
[b] Titers are expressed as the $\log_{10}$ of the highest dilution of MAb which showed specific fluorescence by MIF assay or which neutralized more than 50% of the infectivity of strain AB7.
[c] —, no reaction observed on Western blot.

Analysis of polyclonal monospecific rabbit sera cross-reactions between the 110-kDa protein and MOMP. A good im-

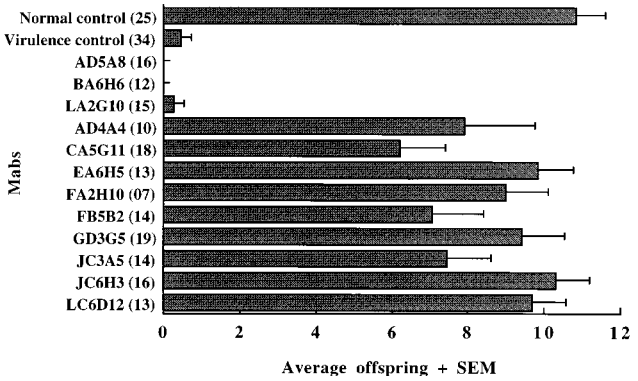

FIG. 1. Protective effects of MAbs on infected pregnant mice as evaluated by the average number of live baby mice per litter ± standard error of the mean (SEM) 8 days after birth. The normal control group was neither infected nor immunized, and the virulence control group was infected but not immunized. The number of mice tested in each group is indicated parenthetically.

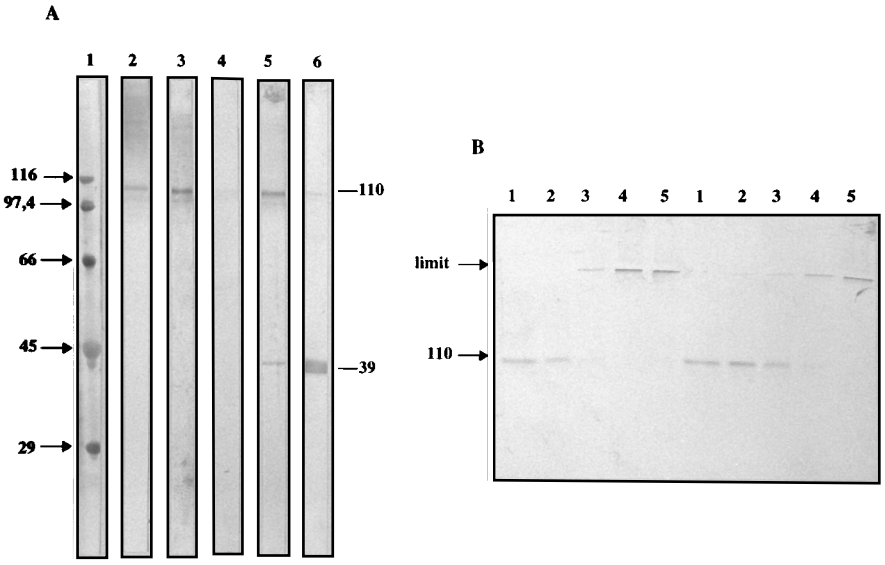

FIG. 2. (A) Immunological cross-reactions between the 110-kDa protein and MOMP. Total chlamydial proteins were heated (lanes 4 and 6) or not heated (lanes 2, 3, and 5). Lanes: 1, molecular mass markers (in kilodaltons); 2, reaction with the protective MAb FA2H10; 3 and 4, reactions with a rabbit polyclonal monospecific serum against the 110-kDa protein; 5 and 6, reactions with a rabbit polyclonal monospecific serum against MOMP. (B) Western blot analysis of the 110-kDa protein with the MAb FA2H10. An extract of total chlamydial proteins was used. Proteins were treated overnight at 4°C in Laemmli buffer with the following percentages of reducing agent β-mercaptoethanol (first five lanes) or dithiothreitol (second five lanes) (only the upper part of the blot is shown): 5% (lanes 1), 0.5% (lanes 2), 0.05% (lanes 3), 0.005% (lanes 4), and 0.0005% (lanes 5). An arrow indicates the limit between the stacking gel and the resolving gel.

mune response was obtained with the two injected proteins. Sera were used at a dilution of 1:1,000 for Western blot analysis (Fig. 2A). The 110-kDa-specific antisera recognized a band only when proteins were not heated; it did not recognize MOMP when the sample was heated before being loaded. Although anti-MOMP sera recognized MOMP only when proteins were heated, it also gave a response at 110 kDa when proteins were not heated.

Affinity purification of the 110-kDa protein. Protein was purified by immunoaffinity with one of the protective MAbs (FA2H10) and eluted with a buffer which allowed the conservation of its immunoreactivity. Separation by SDS-PAGE and subsequent silver staining (Fig. 3A) showed only one band with an apparent molecular mass of 78 kDa. After being heated at 100°C, this protein migrated to the same position as that of MOMP. The heat-denatured protein stained better than did the 78-kDa protein. Western blot analysis (Fig. 3B) showed that the 78-kDa band is recognized by the MAb used during immunopurification and that the 39-kDa band obtained by heat denaturation is recognized by anti-MOMP rabbit sera. The unheated purified protein is also recognized by anti-110-kDa rabbit sera and by anti-MOMP rabbit sera. Heated and unheated purified control proteins incubated with the biotin-streptavidin revelation system without MAb did not show any reaction.

N-terminal amino acid sequencing of the purified protein. Microsequencing of the first 10 N-terminal amino acids of the purified 78-kDa protein revealed the sequence (from 1 through 10) Leu-Pro-Val-Gly-Asn-Pro-Ala-Glu-Pro-Ser, which corresponds to the sequence of the MOMP of the abortion strain S26/3 of *C. psittaci* (14, 30).

DISCUSSION

Of the 28 selected MAbs against the AB7 abortive strain of *C. psittaci*, 19 of diverse specificities neutralized infectivity in vitro. The diversity observed among neutralizing MAbs was also described by Ando et al. (2), who found neutralizing MAbs which differed in isotypes and specificities and recognized different molecules.

In most cases the epitopes recognized by neutralizing MAbs are surface exposed. This statement was confirmed here since recent electron microscopy studies with these MAbs showed strong immunogold labelling on the surfaces of reticulate bodies and elementary bodies (data not shown).

However, only 9 serotype 1-specific MAbs of the 19 neutralizing MAbs were able to protect pregnant mice from abortion after passive antibody transfer assays. These protective MAbs, in contrast to those MAbs which were only neutralizing, had identical specificities, isotypes, and recognized proteins. Despite these similarities, differences among the neutralizing and protective abilities of these MAbs were observed; this could have been the result of different specific MAb concentrations, of different affinities, or of the presence of different epitopes on the same protein.

The unusual amount of IgG2a isotype obtained could have been due to the method used for immunization. It has been shown by Buzoni-Gatel (7a) that intraperitoneal immunization with live abortive chlamydiae, the method used here for the production of MAbs, induces the stimulation of TH1 cells, which in turn produce gamma interferon and interleukin-2. Both of these cytokines favor the production of IgG2a by lymphocyte B cells.

The homology in recognized proteins is indicated by Western blot analysis; all of the protective MAbs reacted with only one protein, the 110-kDa protein. The recognized epitopes are presumably conformational since reactivity in Western blot is lost after heating the proteins. However, Western blot analysis cannot demonstrate that the MAbs recognize the same epitope. The production of MAbs directed to a conformational epitope was probably due to the use of live chlamydiae for immunization, which allows the conservation of native protein structures, and to screening by the MIF method, which allows the selection of antibodies recognizing such epitopes. By immunizing mice with ethylenimine-inactivated chlamydiae, serotype 1-specific protective MAbs (1, 8) were also obtained.

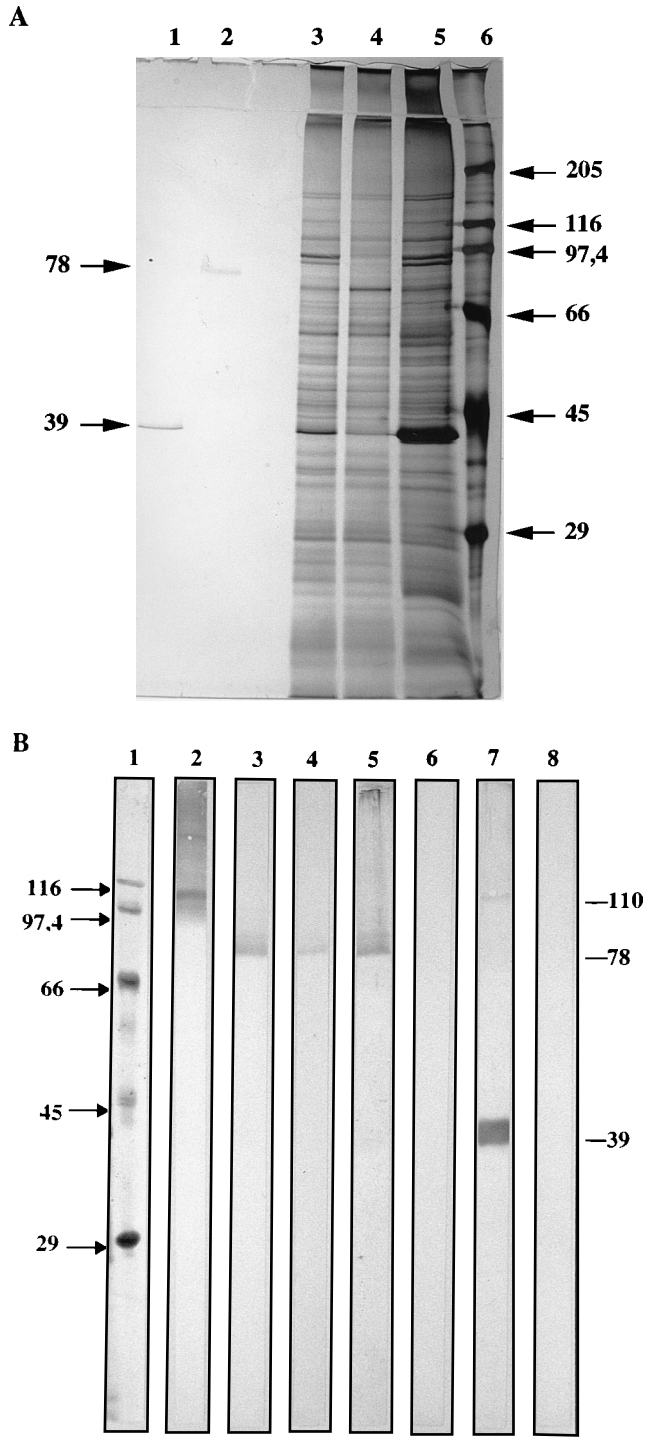

FIG. 3. Analysis of the 110-kDa protein after immunopurification. (A) Silver staining analysis. Lanes: 1, purified and heated protein; 2, purified but unheated protein; 3, total chlamydial proteins after heating; 4, total chlamydial proteins unheated; 5, total chlamydial proteins after heating (the concentration was increased to see the characteristic MOMP pattern); 6, molecular mass markers (in kilodaltons). (B) Western blot analysis. Proteins were heated (lanes 7 and 8) or not heated (lanes 2 through 6). Lanes: 1, molecular mass markers (in kilodaltons); 2, reaction of total chlamydial proteins with the protective MAb FA2H10; 3, reaction of the same MAb with the purified protein; 4, reaction of the purified protein with a rabbit polyclonal monospecific serum against the 110-kDa protein; 5 and 7, reactions of the purified protein with a rabbit polyclonal monospecific serum against MOMP; 6 and 8, control reactions of the purified protein with biotinylated anti-mouse whole IgG and subsequently with a streptavidin-alkaline-phosphatase conjugate.

Nevertheless, inactivation by formalin (11) or β-propiolactone (32) treatment favored the production of genus-specific MAbs, probably because most of the serotype-specific epitopes were destroyed by these inactivants.

Several observations led us to ask if the 110-kDa protein was an oligomer of MOMP. The importance of MOMP for protection has been shown with *C. trachomatis* (36); the existence of MOMP oligomers with molecular masses of about 110 kDa has been shown by chemical cross-linking experiments (6); MOMP was observed by standard SDS-PAGE only after the chlamydial proteins had been heated, whereas the 110-kDa protein was not observed. As MOMP (18), the 110-kDa protein contains cysteine residues since it is able to form large disulfide-linked polymers with other proteins. These polymers did not migrate onto a 10% acrylamide gel until sufficient quantities of reducing agent were added.

To verify the hypothesis of a MOMP polymer, we produced polyclonal monospecific rabbit sera against the 110-kDa protein and MOMP. The reaction obtained between the anti-MOMP sera and the 110-kDa protein was in accordance with the hypothesis of a MOMP oligomer, although the reciprocal reaction was not observed.

Moreover, in order to confirm a possible link between MOMP and the 110-kDa protein, we purified the 110-kDa protein by immunoaffinity with one of our protective MAbs (FA2H10) and compared its migration before and after being heated with that of MOMP. A molecular mass of 78 kDa was obtained for the unheated purified protein (instead of the initial 110-kDa molecular mass). This change can be explained as the result of ultrafiltration washes, which could have eliminated contaminating lipopolysaccharide (LPS) or proteins in interaction with the initial immunoreactive material. Another possible explanation is a conformational modification induced during purification. Furthermore, the first 10 N-terminal amino acids sequenced from the purified protein correspond to those of MOMP from a previously described *C. psittaci* abortion strain (14, 30). Finally, the unheated 78-kDa protein reacted with the protective anti-110-kDa MAb used for purification, with rabbit sera against MOMP, and with rabbit sera against the 110-kDa protein. Heating the sample before analysis allowed the production of only a 39-kDa protein, the molecular mass of MOMP, which reacts only with rabbit sera against MOMP. The lower level of silver nitrate coloration obtained with the 78-kDa protein compared with that of MOMP can be explained by the presence of tertiary and quaternary protein structures (15, 35) in the nondenatured 78-kDa protein.

Taken together, all of these observations argue in favor of a polymer of MOMP forming the 110-kDa protein.

Finally, it has been demonstrated that MOMP monomers have porin activities (4); it is known that native bacterial porins are assembled in noncovalently linked trimers on the outer membrane surface (19). Although disulfide-linked oligomers of MOMP have previously been described (18), the monomers which form the 110-kDa oligomer could be noncovalently linked since the purified oligomer was observed under reducing conditions without the release of MOMP. However, since the apparent molecular mass of 110 kDa is below the expected mass of three linked MOMP monomers, we cannot conclude whether our protein is really a trimer. Nevertheless, the molecular mass observed for nondenatured proteins by SDS-PAGE was only an apparent molecular mass and is very dependent on the protein conformation. Moreover, unheated porin trimers have been found to migrate faster than the expected molecular mass because of their compact shapes (35). The immunological and electrophoretic behavior of this 110-

kDa protein is similar in many other respects to that of native bacterial porin trimers. The more evident observation is that the 110-kDa protein is heat sensitive but SDS resistant, as are native bacterial porins (19). The absence of cross-reactivity between the polyclonal monospecific rabbit antisera to the 110-kDa protein and MOMP is also characteristic of antisera to native bacterial porin trimers, which do not react with denatured monomers (20). Moreover, it has been observed that antisera to porin-denatured monomers can detect the native conformation (26), as did our anti-MOMP sera. Finally, some other bands with higher molecular masses were observed on the Western blot above the protein in its native state; these can be the same protein with different levels of associated LPS, since close relations between LPS and MOMP have previously been demonstrated (6). This association was observed with other porins (5, 21).

McCafferty et al. (17) described a similar 100-kDa protein obtained by a different approach, and their results led them to the same hypothesis of a MOMP trimer. They observed a band at 100 kDa with a MOMP-enriched fraction under nondenaturing conditions. This protein reacted with a MAb against a linear epitope of MOMP, had a sedimentation coefficient very similar to that of bacterial porin trimers, and reacted with the protective MAbs we previously described (8).

The possibility that discontinuous MOMP epitopes play a role in inducing protective immunity has already been suggested by Batteiger et al. (3). Our results, together with those of McCafferty et al., agree with this hypothesis and show the importance of a presumably conformational epitope on a MOMP polymer for protection against abortive strains of *C. psittaci.*

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