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# Systemic and Mucosal Antibody Response in Experimental *Chlamydia pneumoniae* Infection of Mice

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***Chlamydia pneumoniae* is a common human respiratory pathogen, and sera from infected individuals recognize several proteins of *C. pneumoniae*. We produced *C. pneumoniae*-specific proteins in a *Bacillus subtilis* expression system. We then used these recombinant *C. pneumoniae* proteins and purified *C. pneumoniae* elementary bodies as antigens in enzyme immunoassays to assess the kinetics and protein specificity of the systemic and mucosal antibody responses induced by *C. pneumoniae* intranasal infection in BALB/c mice. The systemic antibodies in mice recognized strong 'key' immunogens of *Chlamydia*, Omp2 and Hsp60, but weakly targeted the MOMP protein, the major immunogen in chlamydial species other than *C. pneumoniae*. The IgA antibodies in bronchial secretions specifically recognized the putative surface protein of *C. pneumoniae*, Omp4. Our preliminary observations point to the necessity of further characterization of the mucosal antibody response during *C. pneumoniae* infection.**

**Abbreviations:** EB, elementary body; EIA, enzyme immunoassay; Hsp, heat shock protein; IFU, inclusion-forming units; Ig, immunoglobulin; MOMP, major outer membrane protein; Omp, outer membrane protein; Pmp, polymorphic membrane protein; SDS, sodium dodecyl sulfate

*Chlamydia pneumoniae* causes acute respiratory infections like pneumonia, bronchitis, and pharyngitis,[15] and it has been associated with several chronic conditions, such as asthma and atherosclerosis.[17,34] *C. pneumoniae* infects primarily respiratory epithelium, but according to in vitro studies, it can infect other cell types (for example, macrophages) as well.[14] Western blotting with acute- and convalescent-phase sera from human patients has identified several *C. pneumoniae*-derived antigens present in elementary bodies (EBs) during human infection,[6,7,10,13,20,33] but consistent antigen recognition patterns indicating acute or persistent *C. pneumoniae* infection have not yet emerged.

We have used a *Bacillus* expression system for production of recombinant *C. pneumoniae* proteins.[1] In our earlier study, we produced and purified *C. pneumoniae* major outer membrane protein (MOMP), outer membrane protein 2 (Omp2), and 60-kDa heat-shock protein (Hsp60) and evaluated their usefulness in characterizing immune responses in a murine *C. pneumoniae* infection model.[1] After challenge and rechallenge, as well as after DNA immunization and subsequent challenge, these *Bacillus*-produced *C. pneumoniae* proteins proved to be useful as enzyme immunoassay (EIA) antigens and antigens in a lymphoproliferation test.[1,31] In the present study, we used 5 recombinant *C. pneumoniae* antigens—4 envelope proteins (MOMP, Omp2, Omp4, and Omp5) and a cytoplasmic chaperone (Hsp60, also known as GroEL)—as well as purified *C. pneumoniae* EBs as antigens in EIAs to further evaluate the long-term kinetics of the systemic antigen-specific antibody response in mice after *C. pneumoniae* infection and rechallenge.

MOMP is probably the best-characterized antigen among chlamydial species. Although MOMP is the predominant immunogen in *C. trachomatis*, human antibodies only weakly target denatured MOMP in *C. pneumoniae* infections.[5,6] In contrast, Omp2, a 62-kDa periplasmic structural protein,[11] is a strong immunogen in all *Chlamydia* species.[27] Further, heat-shock proteins are ubiquitous and abundant proteins; one of their functions is to serve as chaperones to ensure correct folding and conformation of proteins. The possible role of heat-shock proteins in the immunopathology of chronic chlamydial infections is still unknown; high chlamydial Hsp60 antibody titers in humans with pelvic inflammatory disease correlate with severe inflammatory manifestations of the disease.[8,9]

Melgosa and coworkers reported a new cysteine-rich, 98-kDa protein in the outer membrane complex of *C. pneumoniae*.[24] Later, Knudsen and coworkers were the first to identify the *C. pneumoniae* genes encoding proteins of this size (*omp4* and *omp5*),[22] and subsequent analysis of genomic sequences revealed a new family of polymorphic membrane proteins (Pmps) that also includes the aforementioned Omp4 (Pmp11) and Omp5 (Pmp10).[23,35] These proteins presumably are exposed on the surface of the chlamydia organism. The function and the immunogenicity of these proteins are still unknown. However, because *C. pneumoniae's* small genome has open reading frames for 21 Pmps, they are expected to have an important role in the biology and pathogenesis of this organism.[16,35]

Antibodies in local secretions have been suggested to play a role in protection in genital and pulmonary infection by *C. trachomatis* (strain MoPn).[25,26,39] We therefore wanted to characterize the presence and protein specificity of mucosal anti-chlamydial immunoglobulins during primary infection and reinfection by *C. pneumoniae*.

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## Materials and Methods

**Chlamydia.** *C. pneumoniae* Kajaani 6 (K6) isolate was provided by Professor Pekka Saikku (University of Oulu, Finland). *Mycoplasma*-free *C. pneumoniae* K6 was propagated in HL cells. *C. pneumoniae* EBs were separated from infected HL cells as previously described.[30] In brief, the cells were disrupted by sonication, cell debris was removed, and the bacteria were purified through a meglumine diatrizoate gradient, suspended in SPG buffer (0.25 M sucrose, 10 mM sodium phosphate, 4 mM potassium phosphate, 5 mM L-glutamic acid, pH 7.5), and stored at –70 °C until used. The infectivity of the bacterial preparations was determined by counting inclusions in cycloheximide-treated HL-cell cultures and expressed as inclusion forming units (IFU) per ml.

**Antigens.** Purified *C. pneumoniae* EBs in SPG and recombinant *C. pneumoniae* proteins were used as antigens in EIAs. Recombinant *C. pneumoniae* proteins used in this study (MOMP [also known as OmpA; GenBank accession no., M69230],[32] Omp2 [OmcB; accession no., X53511],[38] Hsp60 [GroEL; GenBank accession no., M69217],[21] Omp4 [Pmp11], and Omp5 [Pmp10; GenBank accession no., AJ001311[22]]) were produced in a *B. subtilis* expression system as described earlier.[1] A His-tag was introduced into the C-terminal end of each recombinant protein to facilitate purification by nickel column chromatography (Qiagen, Hilden, Germany). His-tagged Omp4 was purified from the cell particulate fraction under denaturing conditions according to the protocol for MOMP.[1] Omp5-$His_6$ showed poor binding to the nickel resin and therefore was purified after solubilization in sodium dodecyl sulfate (SDS) and β-mercaptoethanol by ion-exchange chromatography (Sepharose CL 6B, Amersham Biosciences, Uppsala, Sweden) in the presence of SDS and β-mercaptoethanol. The purity of the proteins was determined by denaturing polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue.

**Mouse infection.** Specific pathogen-free female BALB/c mice (Bomholtgård Breeding and Research Centre, Ry, Denmark) were housed in ventilated cages, given food and water ad libitum, and used at 6 to 8 wk of age. Metofane-anesthetized mice were infected intranasally with $1 \times 10^6$ IFU of *C. pneumoniae* K6. Infection kinetics were described as the median number of IFUs per lung at each time point, as described previously.[30] The mice have been included in a health monitoring system that follows the guidelines of the Federation of European Laboratory Animal Science Associations, and the mice were free of 6 viruses (minute virus of mice, mouse hepatitis virus, pneumonia virus of mice, reovirus 3, Sendai virus, and Theiler encephalomyelitis virus), 10 bacterial and fungal species (*Clostridium piliforme*, *Bordetella bronchiseptica*, *Citrobacter freundii*, *Corynebacterium kurcheri*, *Mycoplasma* spp., *Pasteurella* spp., *Salmonellae*, *Streptobacillus moniliformis*, β-hemolytic streptococci, and *Streptococcus pneumoniae*), and ectoparasites and endoparasites. The Institutional Ethics Committee on Animal Experimentation of the National Public Health Institute (Helsinki, Finland) and the provincial state office of southern Finland approved all the animal experiments. Mice were euthanized by inhaled $CO_2$ overdose.

**Antibody EIA.** Sera of 6 or 7 mice per time point were collected at indicated time points after primary infection and reinfection. Immunoglobulins (IgG, IgG1, IgG2a, and IgA) in mouse sera were determined by EIA as described earlier, with minor modifications.[31] In brief, polystyrene 96-well plates (Nalge, Hereford, UK) were coated with recombinant *C. pneumoniae* proteins (0.75 to 1 μg/ml) or $1 \times 10^5$ IFU *C. pneumoniae* EBs/well overnight at room temperature. After blocking with 3% bovine serum albumin in phosphate-buffered saline, diluted mouse sera were allowed to bind to the proteins for 1 h at room temperature. For Ig, IgG, and IgG subclass analysis, mouse sera was diluted to 1:100, 1:300, and 1:1000, whereas for serum IgA analysis, dilutions of 1:30, 1:100 and 1:300 were used. The plates were washed with phosphate-buffered saline containing 0.05% Tween 20, and binding was detected with horseradish peroxidase-labeled antibody to mouse Ig (Dako A/S, Glostrup, Denmark), IgG (Sigma–Aldrich, St Louis, MO), IgG subclasses (Binding Site, Birmingham, UK), or IgA (Sigma–Aldrich). After washing, the substrate (BM Blue POD Substrate, Roche Diagnostics, Basel, Switzerland) was added, and the optical absorbance at 450 nm was measured. The EIA results were expressed as logarithmic end-point titers read at OD 450 absorbance value 0.3. For IgA measurements end-point titers were read at OD value 0.2. For calculating median titers for Ig-, IgG-, IgG1-, and IgG2a-specific responses, all titers less than 2 were valued as 1.7, and for IgA responses, all titers less than 1.47 were valued as 1.18.

In addition, *C. pneumoniae* EB- or recombinant protein-specific IgA antibodies in bronchoalveolar lavage (BAL) samples were measured by EIA. BAL samples were collected on days 10, 18, 28, and 42 after primary infection and on days 3, 6, and 18 after reinfection. Mice were euthanized, and the bronchial tract of each was flushed with a total of 1 ml phosphate-buffered saline. BAL fluids were stored at –20 °C. Before their use in EIAs, the BAL samples and supernatants from lung homogenates were centrifuged ($16{,}000 \times g$ for 1 min) and then diluted 1:4, 1:8, and 1:32 in phosphate-buffered saline. Bound IgA was detected by use of horseradish peroxidase-labeled antibody to mouse IgA (Sigma–Aldrich) and BM Blue POD Substrate (Roche). EIA results were expressed as logarithmic endpoint titers at $OD_{450} = 0.2$. For calculating medians, all titers less than 0.6 were valued as 0.3.

## Results

**Antibody response to *C. pneumoniae* bacteria.** Intranasal inoculation of BALB/c mice with $10^6$ IFU *C. pneumoniae* leads to self-limited pulmonary infection.[30] As expected, cultivable bacteria peaked at approximately $10^5$ IFU/lung during the first wk, after which the number of bacteria started to decrease (Figure 1 A). Primary infection conferred partial protection, as faster clearance of bacteria occurred after rechallenge (Figure 1 A). When infected for the first time, mice developed a very mild systemic humoral antibody response against *C. pneumoniae* EBs, as measured by EIA (Figure 1 B). After reinfection, antibody levels markedly increased, and serum IgG was detected throughout the follow-up period of 45 d. Low titers of IgG1 and IgG2a isotypes were detected in sera after primary infection (Figure 1 C), and titers increased after reinfection (Figure 1 D). Neither IgG isotype (IgG1 versus IgG2a) predominated in sera, thus suggesting a mixed Th1/Th2-type immune response during infection.

Serum IgA antibodies against *C. pneumoniae* EBs became measurable at 11 d after rechallenge (Figure 1 E). Although a serum IgA response was not detectable after the primary infection, *C. pneumoniae* EB-specific mucosal IgA were detected readily in BAL specimens from infected mice (Figure 2). In BAL samples, IgA antibodies appeared at 10 d after infection and remained detectable at all later time points tested (as long as 6 wk after infection). Because neither an IgA response in serum nor IgG in BAL fluids were detected with the same assay after primary challenge (except from 1 mouse at day 10 after primary infection [data not

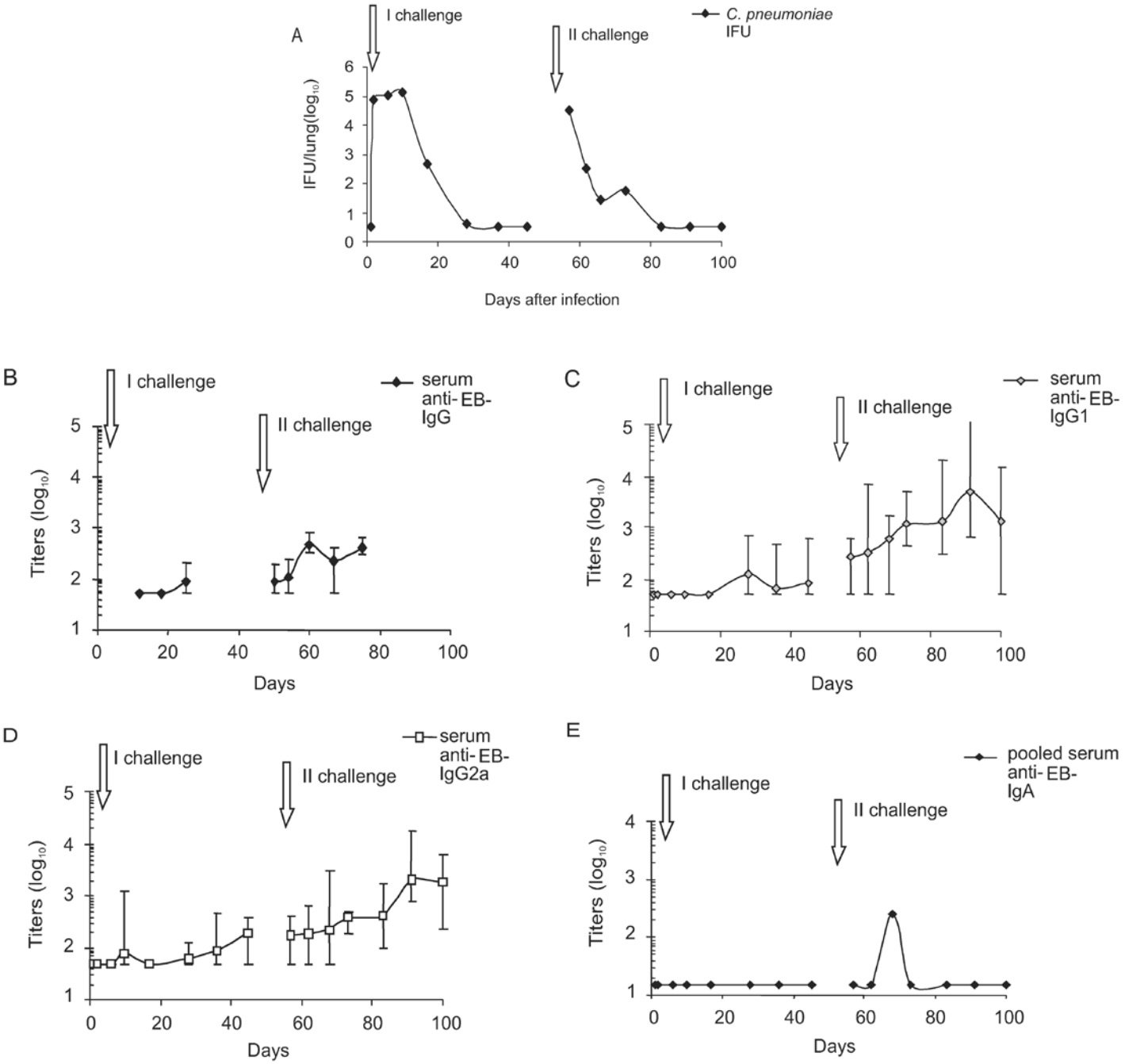


**Figure 1.** Pulmonary bacterial load (A) and serum IgG (B), IgG1 (C), IgG2a (D), and IgA (E) antibody response to *C. pneumoniae* elementary bodies (EBs) in BALB/c mice during primary infection and reinfection with *C. pneumoniae*. Mice were intranasally infected with $10^6$ inclusion-forming units (IFU) of *C. pneumoniae* K6 isolate, as described previously.[36] Sera were collected from the mice at indicated time points after primary infection (I challenge) and reinfection (II challenge). Results are expressed as mean logarithmic endpoint titers (bar, range) read at $OD_{450} = 0.3$. For IgA measurements, endpoint titers were read at $OD_{450} = 0.2$. For calculating mean titer for Ig, IgG, IgG1, and IgG2a specific responses, all titers less than 2 were valued as 1.7; for IgA responses, all titers less than 1.47 were valued as 1.18. No antibodies (titer, <2) were detected in sera from uninfected mice.

shown]), the IgA antibodies detected in BAL fluid most likely consisted of secretory IgA rather than IgA exuded from the blood circulation. Mucosal IgA levels were elevated during reinfection, and the induction was more rapid after rechallenge than after the first infection. In addition, mucosal IgA levels and pulmonary bacterial counts in infected mice were inversely correlated. Such correlation was less pronounced between pulmonary bacterial counts and serum antibodies.

**Antibody response to recombinant *C. pneumoniae* proteins.** We then analyzed the antigen specificity of both serum and mucosal antibodies. We used 5 *C. pneumoniae* recombinant proteins—MOMP, Omp2, Hsp60, Omp4, and Omp5—produced in *B. subtilis* as EIA antigens[1] and evaluated the humoral response to these proteins and the long-term kinetics of that response in *C. pneumoniae*-infected mice. A marked Omp2 antibody response appeared in sera already at 10 d after primary infection. Antibod-

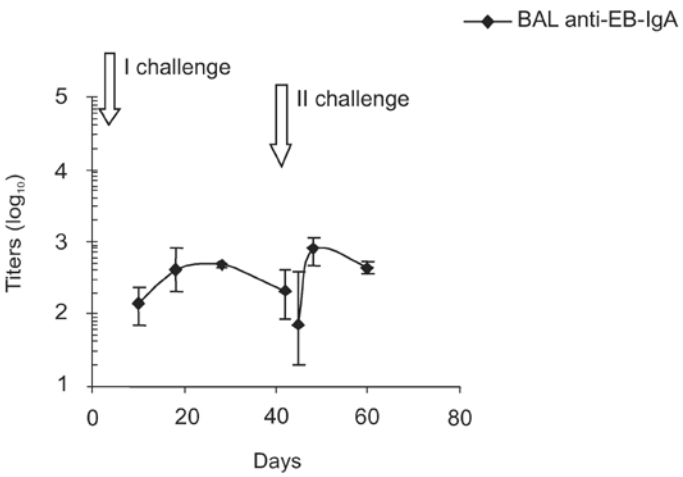


**Figure 2.** Pulmonary IgA response to *C. pneumoniae* elementary bodies (EBs) in mice during primary and reinfection with *C. pneumoniae*. Mice were infected intranasally as described in the legend to Figure 1. Bronchoalveolar lavage (BAL) samples were taken on days 10, 18, 28, and 42 after primary infection (I challenge) and on days 3, 6, and 18 after reinfection (II challenge). Results are expressed as mean logarithmic endpoint titer (bar, range) at $OD_{450} = 0.2$. For calculating means, all titers less than 0.6 were valued as 0.3. No antibodies (titer, <0.6) were detected in BAL samples from uninfected mice.

ies recognizing *C. pneumoniae* MOMP or Hsp60 could be detected only at low levels after 2 wk of infection (Figure 3 A). Antibodies against Omp4 and Omp5 were almost undetectable after primary infection (Figure 3 B). After reinfection, *C. pneumoniae* Hsp60, in addition to the Omp2 protein, became a prominent immunogen recognized by serum antibodies (Figure 3 A). Rechallenge induced a rapid increase in Hsp60-specific antibodies that decreased slightly and then remained at steady-state levels until the end of follow-up (approximately 3 mo). Omp2 antibody levels were already high at the time of rechallenge and remained high throughout reinfection. Upon rechallenge, MOMP-, Omp4-, and Omp5-specific antibodies could be detected by EIA, but the levels in serum remained low (Figure 3 A, B).

Mucosal secretory IgA antibodies are involved in inhibition of microbial adhesion to host cells and therefore recognize antigenic structures on the surfaces of microbes. After primary infection, the only antigen in our 5-protein panel that was recognized by IgA antibodies in BAL was Omp4 (Figure 4 B), whereas Omp2 was the main antigen recognized by serum antibodies (Figure 3 A). However, after reinfection, production of Omp2- and Hsp60-reacting mucosal IgA antibodies was induced in addition to the Omp4 response. BAL IgA antibodies did not react with MOMP or Omp5 during either primary infection or reinfection with *C. pneumoniae*.

## Discussion

In this study, we confirmed that *B. subtilis* is a suitable host for production of chlamydial proteins for use as antigens in studying humoral immune responses. Sera from infected humans recognize several proteins of *C. pneumoniae*.[6] However, human antibodies only weakly target *C. pneumoniae* MOMP in its denatured form.[5,6] Similarly, the denatured recombinant MOMP antigen, the only form of purified protein available, was relatively weakly recognized by murine antibodies in BAL and sera. This finding

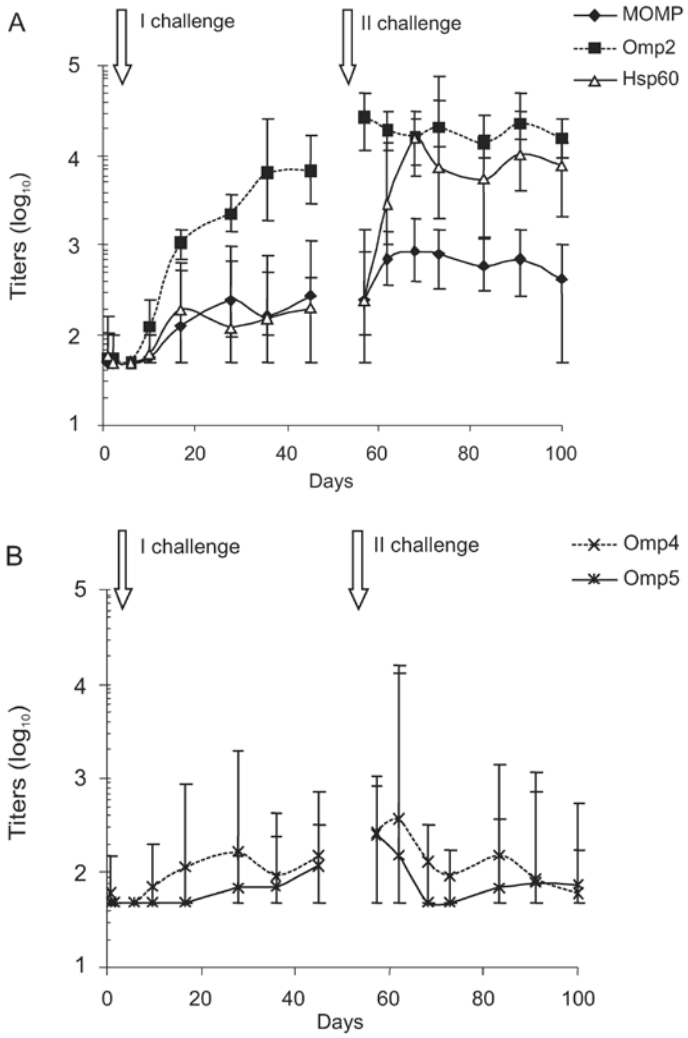


**Figure 3.** Protein-specific serum antibodies in *C. pneumoniae*-infected BALB/c mice. (A) Anti-MOMP-Ig, anti-Omp2-Ig, and anti-Hsp60-Ig. (B) Anti-Omp4-Ig and anti-Omp5-Ig. Mice were infected intranasally as described in the legend to Figure 1. Protein-specific antibodies were measured at various time points; results are expressed as described for Figure 1. I challenge, primary infection; II challenge, reinfection. No antibodies (titer, <2) were detected in sera from uninfected mice.

may reflect structural differences among the outer membranes of different chlamydial species: *C. pneumoniae* MOMP may not be surface-exposed, or the epitopes that are surface-exposed might represent nonlinear conformations.[40] However, sera from mice immunized with denatured *C. pneumoniae*[1] or infected with *C. trachomatis* MoPn strain reacted strongly with this recombinant *C. pneumoniae* MOMP (data not shown). We suggest that the MOMP-specific antibodies that are induced during chlamydial infections recognize both conformational and linear epitopes but that the conformational epitopes may not react with the denatured recombinant proteins used as antigens in serologic assays. In *C. pneumoniae* MOMP, the critical antigenic epitope(s) seem to be nonlinear, whereas linear epitopes may predominate in *C. trachomatis* MOMP.[40]

In contrast, Omp2 (a 62-kDa protein), even in its denatured form, was readily recognized by antibodies present in sera from mice infected once previously. This protein is heavily cross-linked

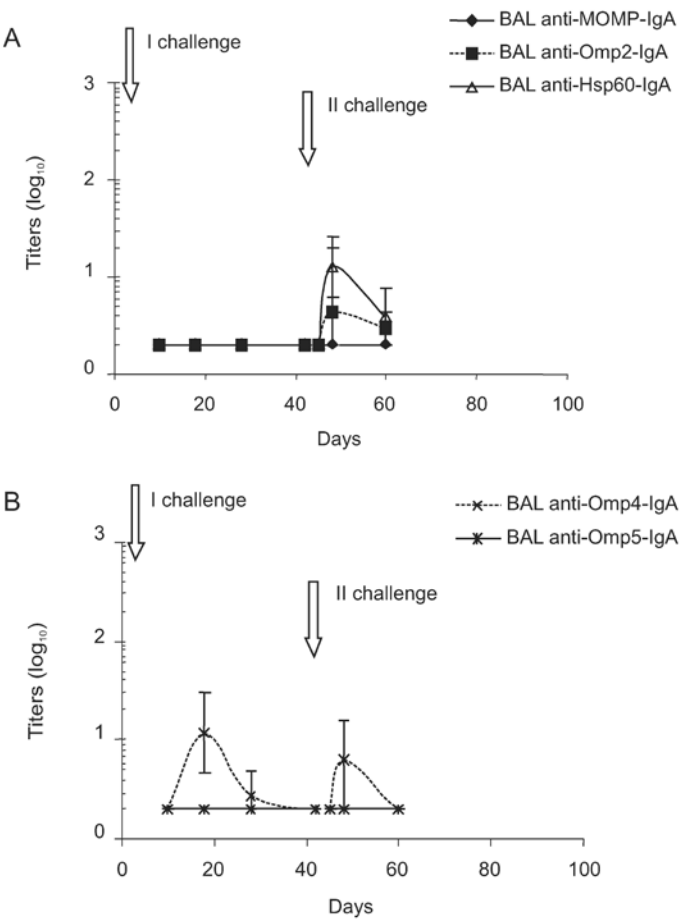


**Figure 4.** Protein-specific pulmonary IgA response in *C. pneumoniae*-infected BALB/c mice. (A) Anti-MOMP-IgA, anti-Omp2-IgA, and anti-Hsp60-IgA. (B) Anti-Omp4-IgA and anti-Omp5-IgA. Protein-specific antibodies were measured at various time points; results are expressed as described in Figure 2. No antibodies (titer, <0.6) were detected in BAL samples from uninfected mice.

by disulfide bonds in EB forms of *Chlamydia* and probably is located in the periplasmic layer, in the close association with the outer membrane.[11,27] In addition, a strong antibody response against Omp2 has been detected in sera from mice immunized with a DNA plasmid coding for *C. pneumoniae* Omp2 as well as with a viral vector expressing the Omp2 protein and in sera from infected humans.[27]

In addition to Omp2, Hsp60 was a predominant humoral immunogen during experimental infection with *C. pneumoniae*. After the 2nd infection, the humoral response to Hsp60 appeared promptly, and the systemic antibody response prevailed at all time points examined (for up to 3 mo). Heat-shock proteins are ubiquitous and abundant proteins that function as chaperones. However, the precise mechanism by which heat-shock proteins are involved in the immunopathology observed after infection is not yet known. In *C. pneumoniae* infection, Hsp60 and Hsp10 antibodies were more prevalent in asthmatic adults than their healthy controls,[3,19] and Hsp60 antibody levels correlated with the presence of *C. pneumoniae* antigen in atheromas.[12] However, *C. trachomatis*, another human chlamydial pathogen that is a leading cause of sexually transmitted disease worldwide, can express Hsp60 during chronic infection.[2] Antibodies against both chlamydial and human Hsp60 often are observed in chronic conditions, suggesting that an autoimmune component plays a role in the pathogenesis of chlamydial infection.[9,10,28]

A 98-kDa cysteine-rich protein in the *C. pneumoniae* outer membrane complex[24] was immunogenic in *C. pneumoniae* human infections.[6] The proteins presumably are surface-exposed, but the pathogenic significance of this PmP family has been unknown. We expressed 2 of these Pmps (Omp4 [Pmp11] and Omp5 [Pmp10]) in *Bacillus*, and Omp4 was the only antigen of our panel that was recognized by mucosal antibodies during primary infection. No Omp4 antibody could be detected in sera after primary infection or repeated infection. Surprisingly, we detected no antibody response against the denatured Omp5 antigen, another polymorphic membrane protein, during mouse infection, although in vitro studies have suggested that Omp5 is one of the more abundant surface proteins of *C. pneumoniae*.[29,36,37] However, Omp5 is differentially expressed in vitro in *C. pneumoniae*-infected Hep-2 cells.[29] Interestingly, Birkelund and coworkers[4] reported that when used to stain lung sections of *C. pneumoniae*-infected C57BL/6 mice, Omp4-specific antibodies reacted with *C. pneumoniae* inclusions but Omp5-specific antibodies did not, although both polyclonal antisera were capable of detecting *C. pneumoniae* in HeLa cells infected in vitro. Consequently, those authors suggested that protein expression of *C. pneumoniae* Omp5 was shut off in infected epithelial cells in mice. In our studies, the absence of antibodies that recognize the Omp5 antigen indeed could be a marker for negligible or no expression of *C. pneumoniae* Omp5 protein in vivo. However, another equally relevant explanation here is inability of denatured recombinant Omp5 antigen to react with mouse antisera. Which serum antibodies recognize specific Pmp proteins induced during acute or persistent *C. pneumoniae* infection in man are unknown. The observed presence of local IgA antibodies to Omp4 in BAL of *C. pneumoniae*-infected mice is consistent with surface exposure of this protein during pulmonary infection. In addition, the increasing IgA antibody levels in local secretions coincident with diminishing bacterial counts is important, because the local antibody response has a protective role during both genital and pulmonary *C. trachomatis* infection.[25,26,39]

The detection of murine and human antibodies that recognize nonlinear epitopes is indeed challenging with assays that use recombinant antigens. However, even by using a panel of only 5 *C. pneumoniae* proteins, we were able to reveal in part the protein specificities of the local and systemic murine antibody responses that were detected with the whole EB antigen. Our preliminary observations suggest that, similar to the situation for murine experimental infection, serum antibody responses to Hsp60 and Omp2 during human chlamydial infection could be detected with EIA using recombinant proteins.[18,19] However, specific assays for chronic *C. pneumoniae* conditions are highly desired but do not exist yet. The need for a broader protein panel for measuring antigen-specific responses in humans with acute and persistent conditions should be addressed. In particular, the possible role of the local IgA response in humans and the antigen specificity of the antibodies involved warrant further study.

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