Analysis of the Humoral Immune Response to Chlamydia pneumoniae by Immunoblotting and Immunoprecipitation

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Chlamydia pneumoniae is a widely spread agent of respiratory tract infections in humans. A reliable serodiagnosis of the disease is hampered by the poor knowledge about immunodominant antigens in C. pneumoniae infections. We applied a novel strategy to identify immunogenic proteins of C. pneumoniae TW183 combining metabolic radiolabeling of de novo-synthesized chlamydial antigens with immunoprecipitation. By this technique C. pneumoniae antigens of approximately 160, 97 to 99, 60 to 62, 40, 27, and 15 kDa were detected in the vast majority of sera from patients with a current C. pneumoniae infection. By immunoblotting purified elementary bodies of C. pneumoniae TW183 with the same sera, only the 60- to 62-kDa antigen could be detected consistently. Sequential immunoprecipitation performed at different stages of the chlamydial developmental cycle revealed that the 60- to 62-kDa antigen is strongly upregulated after 24 to 48 h of host cell infection and is presented as a major immunogen in both C. pneumoniae-infected patients and mice. We conclude that, due to its high sensitivity and concurrent preservation of conformational epitopes, metabolic radiolabeling of chlamydial antigens combined with immunoprecipitation may be a useful method to reveal important immunogens in respiratory C. pneumoniae infection which might have been missed by immunoblot analysis.

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Chlamydia pneumoniae, an obligate intracellular human pathogen, causes infections of the respiratory tract such as sinusitis, pharyngitis, bronchitis, and pneumonia (15, 22, 25). Seroepidemiological studies showing antibody prevalence rates in a range of 50 to 70% suggest that C. pneumoniae is widely distributed and that nearly everybody is infected with the agent at some time (25, 38). C. pneumoniae is currently of considerable interest because of its link to atherosclerosis, although it still remains unclear whether the organism plays a role as an etiological agent or only as a bystander (20, 24, 39, 45).

Laboratory diagnosis of C. pneumoniae infection is frequently based on serology because (i) cultivation of these fastidious organisms is not routinely possible and (ii) detection of C. pneumoniae DNA is not well standardized and sufficiently evaluated, compared to DNA detection for the urogenital pathogen Chlamydia trachomatis (1). Although the reactive antigen is still unknown, the microimmunofluorescence (MIF) test is widely accepted as the “gold standard” in C. pneumoniae serodiagnosis. However, concern has been raised about its sensitivity and specificity (14, 18, 26). In addition, performance of the MIF assay is time-consuming, and interpretation of the results depends significantly on the investigator’s experience. Therefore, an assay based on defined antigens could be an important improvement in C. pneumoniae serodiagnosis.

Unfortunately, there is only poor knowledge about immunogenic C. pneumoniae proteins, which are recognized consistently by sera of infected individuals. Especially the immunogenic role of the 40-kDa major outer membrane protein (MOMP) has been discussed controversially. According to some immunoblot studies, the MOMP is believed to be weakly immunogenic (2, 7, 26), while in other papers the MOMP was characterized as an immunodominant protein (19, 21). The 60-kDa cysteine-rich outer membrane protein 2 (OMP2), a structural protein of the chlamydial outer membrane complex (OMC), contains genus-reactive epitopes and seems to be a major immunogen in both human C. pneumoniae and C. trachomatis infections although it is probably not surface exposed (32, 34, 43). An artificial glycoconjugate antigen has been used to develop an enzyme-linked immunosorbent assay measuring antibodies against the chlamydial lipopolysaccharide (LPS), which has been characterized as a major surface antigen of chlamydial organisms (4, 5, 27). Further antigens with molecular masses of 98, 68, 60, 53, 43, 35, and 30 kDa (8, 12, 19, 21) were detected by Western blot studies, but reactivities differed significantly. In a recent immunoblot study no specific band pattern in terms of reactivity to various C. pneumoniae proteins could be determined (26). In this paper we focused on the C. pneumoniae prototype strain TW 183 and selected a panel of sera from patients with both culture- or PCR-proven respiratory C. pneumoniae infection and serological evidence for C. pneumoniae infection according to recommended criteria (25).

A novel approach was applied to determine immunodominant antigens in human C. pneumoniae infection. Metabolic labeling of de novo-synthesized antigens from different chlamydial developmental stages was combined with immunoprecipitation, a method which enables sensitive detection of reactive antigens without affecting their conformational epitopes. Based on the band patterns of precipitated antigens visualized by autoradiography, we propose a profile of C. pneumoniae antigens which are consistently recognized by sera from C. pneumoniae-infected individuals.

MATERIALS AND METHODS

Bacterial strain. C. pneumoniae TW 183 (Washington Research Foundation, Seattle, Wash.) was used throughout this study and maintained continuously on cycloheximide-treated HeLa 229 cell monolayers (American Type Culture Collection; CCL 2.1) in six-well culture plates by standard procedures. Glass coverslips placed into the culture plates were stained by the fluorescent-antibody technique with a Chlamydia genus-specific mouse monoclonal antibody (Pathfinder, Chaska, Minn.) to determine the percentage of infected host cells by counting the inclusion-forming units (IFU) under a fluorescence microscope. For immunoblot analysis chlamydial elementary bodies were purified by urea/gradient centrifugation as described previously (40). For radiolabeling and immunoprecipitation, cultures with at least 80% infected host cells were harvested after 72 h and homogenized with glass beads. After brief centrifugation
TABLE 1. Characterization of patient sera used and their main activities addressed with purified C. pneumoniae TW 183 elementary bodies

| Patient (lane in Fig. 1) | Age/sex | Clinical picture (wk) | Chlamydia detection method | MIF titer | IgG immunoblot reactivity for band |
|--------------------------|---------|-----------------------|-----------------------------|-----------|----------------------------------|
| Rei (1)                  | 43/M    | Persistent cough (10) | Culture                     | 40        | 2,048                            |
| Sml (4)                  | 38/F    | Sinusitis, bronchitis (4) | PCR                        | <20       | 1,024                            |
| Rch (3)                  | 39/F    | Pneumonia (7)         | PCR                        | 20        | 512                             |
| Wern (4)                 | 61/F    | Pneumonia, pneumonia (4) | PCR                        | <20       | 2,048                           |
| Kal (5)                  | 14/F    | Pneumonia (14)        | PCR                        | 80        | 128                             |
| Det (6)                  | 28/F    | Bronchitis (4)        | PCR                        | 80        | 1,024                           |
| Kle (7)                  | 12/M    | Pneumonia (4)         | PCR                        | 80        | 1,024                           |
| Scd (8)                  | 39/F    | Bronchitis (4)        | PCR                        | 80        | 1,024                           |
| S 11 (9)                 | 45/F    | Persistent cough (26) | PCR                        | 80        | 1,024                           |
| S 38 (10)                | 15/M    | Pharyngitis (16)      | Culture                    | <20       | 512                             |

| Fig. 1B                  |         |                       |                             |           |                                  |
| Dik (11)                 |         | Cervicitis, bronchitis (4) | Culture                 | <20       | 1,024                           |
| Swa (12)                 |         | Urethritis            | LCR<sup>a</sup>          | <20       | 128                             |
| Bir (13)                 |         | Ornithosis (8)        | Culture                    | 40        | 1,024                           |
| Hbd (14)                 |         | Healthy blood donor   | ND<sup>b</sup>            | <20       | 64                              |

<sup>a</sup> M, male; F, female.
<sup>b</sup> Weeks after onset of symptoms (?), onset unknown.
<sup>c</sup> Reciprocal of the serum dilution from the MIF test for C. pneumoniae.
<sup>d</sup> Bands were scored by visual comparison with standard sera (100, 200, 400, 800, 1600, 3200, 6400, 12,800, 25,600, 51,200, 102,400, 204,800). Titers of sera were not determined.
<sup>e</sup> Ten sera from patients with C. pneumoniae infection.
<sup>f</sup> Serum from a patient (lane 11) with culture-positive C. trachomatis cervicitis and suspected C. pneumoniae bronchitis, serum (lane 12) representative of 10 sera from patients with urogenital C. trachomatis infection, serum from a patient with ornithosis (lane 13), and serum (lane 14) representative of 10 sera from healthy adult donors with IgG antibodies, as determined by MIF test, ranging from <32 to 128.

**Immunoblot analysis.** After two washes in 0.22 M sucrose–10 mM NaH<sub>2</sub>PO<sub>4</sub>–3.8 mM KH<sub>2</sub>PO<sub>4</sub>–5 mM glutamic acid (pH 7.4), the purity of C. pneumoniae elementary bodies was controlled by immunofluorescence microscopy. Chlamydial proteins (10 µg per lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% polyacrylamide gel system according to the method of Laemmli (28). To absorb nonspecific bindings due to cross-reactivities to potentially contaminating host cell proteins in the sample, all sera used for immunoblot analysis were preincubated with HeLa 229 cell lysates at 4°C overnight. In addition, a sample of homogenized HeLa 229 cells with the same protein weight was run in every separation as a control to detect remaining nonspecific bindings which were not removable by preabsorption. After separation, proteins were transferred to an Immobilon polyvinylidene difluoride transfer membrane (Millipore). Membranes were blocked with a solution of 3% nonfat dried milk from bovines (Sigma Chemicals, Deisenhofen, Germany) for 2 h and incubated with human C. pneumoniae antisera. After being washed with Tris-buffered saline containing 0.05% Tween 20, pH 8.0, the blots were incubated with goat anti-human IgG conjugated to horseshad peroxidase (Sigma Chemicals). Color development was observed on the addition of H<sub>2</sub>O<sub>2</sub> and 3,3-diaminobenzidine tetrahydrochloride (Sigma Chemicals) and stopped by rinsing the blots in H<sub>2</sub>O.

**Cell infection, radioactive labeling, and immunoprecipitation.** C. pneumoniae TW 183 was added to HeLa 229 cell monolayers to give approximately a multiplicity of infection of 10, and the monolayers were centrifuged at 1,600 × g for 1 h. After incubation for 1 h at 37°C in 5% CO<sub>2</sub>, supernatants were replaced by Chlamydia infection medium (growth medium supplemented with cycloheximide [2 µg/ml] and antibiotics [vancomycin at 100 µg/ml and gentamicin at 50 µg/ml]). This time was defined as time zero. Chlamydial protein synthesis was determined between 0 and 24, 24 and 48, and 48 and 72 h of the C. pneumoniae developmental cycle in HeLa 229 cells. Radioactive labeling and immunoprecipitation were performed as described previously (41) with slight modifications. Briefly, sets of 1 × 10<sup>3</sup> to 2 × 10<sup>3</sup> infected and noninfected cells were washed two times with Chlamydia infection medium without l-methionine and l-cysteine. Cells were pulsed with [35S]methionine and [35S]cysteine (250 µCi; PRO-MIX; Amersham) for 24 h at 0, 24, and 48 h after infection. To suppress host cell protein synthesis, a cycloheximide concentration of 50 µg/ml of medium was chosen during the period of pulsing. After being pulsed, adherent host cells were washed three times with phosphate-buffered saline, pH 7.4, to remove [35S]methionine and [35S]cysteine which had not been incorporated by host cells. Bacterial proteins were extracted by treating cells with lysis buffer under the protection of leupeptin, aprotinin, and 4-(2-aminoethyl)benzenesulfonyl fluoride (Calbiochem-Novabiochem, Bad Soden, Germany). Lysates were briefly centrifuged at 19,000 × g, and the supernatants containing the [35S]-labeled proteins were stored in...
The majority of sera reveal immunoreactivity to the 60- to 62-kDa antigen when examined by immunoblotting. Western blots of purified elementary bodies with sera from *C. pneumoniae*-infected patients are shown in Fig. 1A, and the most frequent reactivities are summarized in Table 1. Ninety percent of the sera from patients with culture- and/or PCR-confirmed *C. pneumoniae* infection reacted with the 60- to 62-kDa antigen, which most likely corresponds to OMP2 of *C. pneumoniae*. In 60% of the sera (Fig. 1A, lanes 5 to 10) a strong but blurred signal was obtained at approximately 40 kDa, suggesting reactivity with the 39.5-kDa MOMP of *C. pneumoniae*. In 70% of the sera a 43-kDa antigen that migrated as a sharp band near the MOMP was detected. Further reactivities detected by at least 40% of sera were obtained with antigens of approximately 70, 53, and 15 kDa.

Sera from patients with other chlamydial infections may recognize the 60- to 62- and 40-kDa antigens of *C. pneumoniae* as well. *C. pneumoniae* TW 183 elementary bodies were also reacted with sera of humans with other chlamydial infections to control the specificity of the observed reactions. Figure 1B illustrates the results for representative sera. Eighty percent of the sera from 10 patients with culture- and/or PCR-confirmed *C. trachomatis* infection weakly recognized either the 60- to 62- or the 40-kDa antigens of *C. pneumoniae* TW 183 elementary bodies, probably due to well-known genus-specific epitopes of OMP2 and MOMP (an example is given in Fig. 1B, lane 12).

### RESULTS

Strong and dominant reactivities with *C. pneumoniae* OMP2 were seen in a patient with cervicitis who additionally showed clinical signs of respiratory infection with elevated *C. pneumoniae* IgG antibody titers of 1:2048 (Fig. 1B, lane 11) and in a patient with culture-positive ornithosis showing highly elevated MIF-detected IgG antibodies against all three *Chlamydia* species (Fig. 1B, lane 13). Further genus-specific reactivities were seen at 70 kDa, and the presence of the 8- to 10-kDa chlamydial LPS was confirmed by reactivity with MAb S25-23 (data not shown).

The 46-kDa antigen is nonspecific for *C. pneumoniae* infection. Most of the control sera (80%), which were obtained from adult healthy blood donors, as well as patient sera (100%) recognized a 46-kDa antigen (Fig. 1A and Fig. 1B, lane 14). Strong reactivities against the 60- to 62-kDa protein and the 40-kDa MOMP were lacking in all cases. Faint bands at 60 to 62 and 40 kDa indicating weak OMP2 and MOMP reactions, respectively, were only seen in blood donor sera which exhibited MIF test-determined IgG antibody titers of 1:64 and 1:128, suggestive of a past *C. pneumoniae* infection (Fig. 1B, lane 14).

Immunogenic *C. pneumoniae* antigens are synthesized during the middle and late phases of the cycle. Newly synthesized *C. pneumoniae* antigens were radiolabeled and precipitated after 24, 48, and 72 h, covering thereby the complete chlamydial developmental cycle. During the early phase of the developmental cycle (Fig. 2, lanes 2) at best weak signals could be obtained, suggesting that proteins synthesized during the first 24 h of the developmental cycle are of minor immunogenicity or do not incorporate $^{35}$S. An intense staining of de novo-synthesized antigens was found in the middle and late phases of the growth cycle; however, growth-specific bands which appeared exclusively at one distinct phase of the chlamydial developmental cycle could not be detected (Fig. 2, lanes 4 and 6). Similar band patterns were obtained for all sera from patients with *C. pneumoniae* infection. The frequencies of the main reactivities are given in Table 2, and representative autoradiographs of precipitated antigens for sera from patients with culture- or PCR-confirmed *C. pneumoniae* infection are demonstrated in Fig. 2. Analysis of bands which could be detected by at least 80% of the sera during the middle and/or late phase of the cycle reveals a profile of immunogenic *C. pneumoniae* proteins with estimated molecular masses of about 160, 97 to 99, 60 to 62, 40, 27, and 15 kDa (Table 2; Fig. 2). These reactivities might correspond to previously described proteins of the chlamydial OMC such as the 97- to 99-kDa OMP4 and OMP5, the 60- to 62-kDa OMP2, the 40-kDa MOMP, and the 15.5-kDa OMP3. A protein with an estimated molecular mass of approximately 160 kDa has not been described in published reports, while the 27-kDa protein might correspond to the Mip-like protein of *C. trachomatis*.

**Band patterns of sera from infected patients and healthy blood donors differ significantly.** By analogy with immunoblot analysis, weak reactivities at 40 and 60 to 62 kDa were only obtained with sera of healthy blood donors when sera exhibited MIF test-determined IgG antibody titers of at least 1:64. Strong signals at 40 and 60 to 62 kDa, as well as reactivities with the 160-, 97- to 99-, 27-, and 15-kDa antigens, were not detected. Both control sera and sera from infected patients usually showed a reactivity in a range of 43 to 46 kDa, with both infected and uninfected cells indicating that this reactivity was nonspecific for *C. pneumoniae* infection (Fig. 3).

**Band patterns of sera from infected patients and BALB/c mice did not differ significantly.** BALB/c mice, which have been used to study cell-mediated immunity in *C. pneumoniae*-induced pneumonia, were infected experimentally with *C.
pneumoniae, and pooled sera from mice with histopathological signs of pneumonia were used for immunoprecipitation of biosynthesized chlamydial proteins. A band pattern similar to the patterns obtained from sera of humans with respiratory tract infection was found (Fig. 4).

**MABs recognize the 40-kDa protein of C. pneumonia.** Two murine MABs which have been demonstrated to be species specific for C. pneumoniae by immunofluorescence recognized the 40-kDa antigen when these antibodies were reacted with chlamydial proteins which were synthesized during the middle and late phases of the growth cycle (Fig. 5). However, no reactivity was found when MABs were reacted with purified elementary bodies in an immunoblot analysis (data not shown), suggesting that conformational epitopes of the MOMP were recognized by these MABs.

**DISCUSSION**

The objective was to identify antigens which are recognized by sera of patients infected with C. pneumoniae. We think that the panel of 10 sera used represents sera of truly infected patients because (i) these patients had typical symptoms, (ii) chlamydiae were detected by culture or PCR, and (iii) MIF

**TABLE 2.** De novo-synthesized C. pneumoniae antigens precipitated by 10 human sera from patients with respiratory C. pneumoniae infection (Table 1; Fig. 1A)

| C. pneumoniae antigen recognized (kDa) | % Reactive sera at indicated stage of chlamydial growth cycle<sup>a</sup> |
|----------------------------------------|-----------------------------|
|                                        | 0–24 h.p.i. | 24–48 h.p.i. | 48–72 h.p.i. |
| ~160                                   | 10          | 80          | 70          |
| 97–99                                   | 0           | 100         | 100         |
| 70                                     | 10          | 30          | 20          |
| 60–62                                   | 30          | 100         | 100         |
| 40                                     | 10          | 100         | 100         |
| 30                                     | 0           | 30          | 30          |
| 27                                     | 0           | 80          | 80          |
| 20                                     | 10          | 70          | 70          |
| 15                                     | 10          | 60          | 80          |

<sup>a</sup>Antigens showing cross-reactivity with eukaryotic host cell antigens are not included. Antigens recognized by at least 80% of the sera are in boldface.

<sup>b</sup>p.i., postinfection.
serology was suggestive of an acute infection according to published recommendations. In order to increase the sensitivity of the detection assay, antigens were labeled metabolically during intracellular growth. Since antigen conformation may affect the reactivity, immunoprecipitation was performed. This procedure enabled us to detect de novo-synthesized proteins. Antigens which do not incorporate $^{35}$S-labeled methionine and cysteine or which are not bound by the respective antibody cannot be detected. This might be an explanation for the lack of bands in specimens of the early growth phase. We did not detect bands which were specific for the second and third growth phases.

Based on the band patterns of autoradiographs from precipitated proteins during the middle and late phases of the developmental cycle, we established a profile of immunogenic proteins from *C. pneumoniae* prototype strain TW 183 which were detected by at least 80% of the sera and which migrated during SDS-PAGE at molecular masses of approximately 160, 97 to 99, 60 to 62, 40, 27, and 15 kDa. Bands with molecular masses of 60 to 62, 40, and 15 kDa were observed in both immunoblotting and immunoprecipitation experiments.

The 60- to 62-kDa antigen, which is strongly upregulated after 24 to 48 h of host cell infection, showed up clearly as a major immunogen in both *C. pneumoniae*-infected patients and mice, as well as in patients infected with other chlamydial species. In previous work it was shown that the cysteine-rich proteins of the chlamydial outer membrane are synthesized late in the cycle when reticulate bodies have begun to reorganize back to elementary bodies (11, 16, 33). In contrast, the 60-kDa chlamydial GroEL incorporates $^{35}$S early (2 to 8 h postinfection) in its biosynthesis, with a decrease of protein synthesis from 26 to 30 h postinfection (29). Based on these observations, we suggest that the 60- to 62-kDa protein detected by all sera in the middle and late phases of the growth cycle probably corresponds to the cysteine-rich OMP2 but not to the GroEL homolog, which also migrates at approximately 60 kDa in SDS-PAGE. The cysteine-rich OMP2 is thought to constitute the structural integrity of chlamydial elementary bodies and contains both sequences and antigenic determinants shared with proteins from other *Chlamydia* spp. (11, 31, 32, 34). Our data confirm and extend findings from Mygind et al., who suggested, on the basis of the reactivity of MIF-defined patient sera to truncated fusion proteins, that the 60- to 62-kDa OMP2 was a major immunogen in both *C. pneumoniae*- and *C. trachomatis*-infected patients (32).

Weak reactivity of patient sera in immunoblotting, failure to establish neutralizing antibodies, and lack of murine MAbs that recognize MOMP by immunoblot analysis have led to the assumption that the *C. pneumoniae* MOMP, at least in its linear form, is not a major target of the humoral immune response in *C. pneumoniae* infection (7, 26, 36). Contradicting results, however, were obtained by others (19, 21). Loss of reactivity by conformational changes and higher sensitivity might explain the detection of the antigen by immunoprecipitation but not by immunoblotting with murine species-specific MAbs. Our findings indicate that the biosynthesized, native 40-kDa MOMP is recognized consistently by sera from *C. pneumoniae*-infected patients and suggest that the native 40-kDa MOMP may be better recognized than the denatured protein.

A 15.5-kDa cysteine-rich protein was found in the OMC of *C. pneumoniae* (31). This protein is comparable in molecular mass to the cysteine-rich OMP3 of 12.5 to 15.5 kDa from *C. trachomatis* (9). The immunogenic role of the 15.5-kDa protein in *C. pneumoniae* infection has not been elucidated until now, most probably because at best only faint bands are detectable by immunoblotting analysis. In our study the detection sensitivity was increased by metabolic radiolabeling of biosynthesized chlamydial proteins. The majority of sera precipitated a protein with a molecular mass of 15 kDa in the middle and/or late phase of the life cycle, indicating that OMP3 of *C. pneumoniae* may be also a target of the humoral immune response in *C. pneumoniae* infection. In addition, bands of approximately 160, 97 to 99, and 27 kDa were detected by metabolic labeling and immunoprecipitation but not by immunoblotting. The 27-kDa antigen could be a homolog to the Mip-like protein of *C. trachomatis*, which is thought to be important for optimal initiation of chlamydial infections (30), while the 160-kDa antigen could correspond to the pmpD-encoded OMP with a predicted molecular mass of 160 kDa; *pmpD* has been identified as a gene in *C. trachomatis* (40).
In a recent paper two novel genes encoding 97- to 99-kDa surface-located OMPs (OMP4 and OMP5) of \textit{C. pneumoniae} have been identified (23). Conformational epitopes of OMP4 seem to be the target of the humoral immune response in experimentally infected mice, since this protein was detectable by immunoblotting only when it was not fully denatured. This is in agreement with our findings, which revealed a 97- to 99-kDa band by immunoprecipitation, but not by immunoblotting. In addition, we could show that this antigen is also immunogenic in naturally infected humans. We conclude from our data that the 60- to 62-kDa protein of \textit{C. pneumoniae} represents a major immunogen in patients with respiratory \textit{C. pneumoniae} infection. In addition, \textit{C. pneumoniae} proteins of 97 to 99, 40, and 15.5 kDa, which most probably correspond to well-characterized components of the chlamydial OMC, along with proteins of approximately 160 and 27 kDa seem to have immunogenic importance. Further work is needed to clarify if some of these antigens are also suitable for a species-specific or for a genus-specific serodiagnostics.

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