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# Immunoproteomic Identification and Serological Responses to Novel *Chlamydia pneumoniae* Antigens That Are Associated with Persistent *C. pneumoniae* Infections[1]

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**The controversial discussion about the role of *Chlamydia pneumoniae* in atherosclerosis cannot be solved without a reliable diagnosis that allows discrimination between past and persistent infections. Using a proteomic approach and immunoblotting with human sera, we identified 31 major *C. pneumoniae* Ags originating from 27 different *C. pneumoniae* proteins. More than half of the proteins represent *Chlamydia* Ags not described previously. Using a comparative analysis of spot reactivity Pmp6, OMP2, GroEL, DnaK, RpoA, EF-Tu, as well as CpB0704 and CpB0837, were found to be immunodominant. The comparison of Ab-response patterns of sera from subjects with and without evidence for persisting *C. pneumoniae,* determined by multiple PCR analysis of PBMC and vasculatory samples, resulted in differential reactivity for 12 proteins, which is not reflected by reactivity of the sera in the microimmunofluorescence test, the current gold standard for serodiagnosis. Although reactivity of sera from PCR-positive donors was increased toward RpoA, MOMP, YscC, Pmp10, PorB, Pmp21, GroEL, and Cpaf, the reactivity toward YscL, Rho, LCrE, and CpB0837 was decreased, reflecting the altered protein expression of persisting *C. pneumoniae* in vitro. Our data provide the first evidence of a unique Ab-response pattern associated with persistent *C. pneumoniae* infections, which is a prerequisite for the serological determination of persistently infected patients. *The Journal of Immunology,* 2008, 180: 5490–5498.**

The respiratory pathogen *Chlamydia pneumoniae* (*Chlamydophila pneumoniae*) occurs worldwide with a seroprevalence up to 70% (1). After primary infection, the obligate intracellular bacteria can persist in the host (2, 3). Persisting *C. pneumoniae* are frequently found in the respiratory tract (4–6) or in atherosclerotic blood vessels (7–11) and therefore represent a potential risk factor for chronic inflammatory lung diseases (12–14) or for atherosclerosis (15). It is assumed that *C. pneum*oniae disseminate from the respiratory tract via infected phagocytes (16, 17), which are found to contain *C. pneumoniae* even in healthy volunteers (18–20).

Although the direct detection of the pathogen in atherosclerotic plaques, as well as animal models, and in vitro data support the hypothesis that *C. pneumoniae* is involved in atherogenesis at some stages (15), confusing results stem from seroepidemiological studies and treatment trials, where the stratification of donors was based only on serologic criteria. The first study of Saikku et al. (21) showing a link between coronary artery disease and serological evidence for a past infection with *C. pneumoniae* was followed by many others, and today the number of reports showing a positive association is similar to the number of reports that show the opposite. These inconsistencies can likely be attributed to the poor validity of current *C. pneumoniae* serodiagnosis (22–25). Although the microimmunofluorescence (MIF)[4] assay—which is based on whole *C. pneumoniae* elementary body (EB)—is considered the "gold standard" for *C. pneumoniae* serology (26), the test suffers from subjective interpretation, cross-reactivity between different *Chlamydia* species, and high intra- as well as interlaboratory variations (22–25, 27). Moreover, it does not correlate with the presence of *C. pneumoniae* in the host (7, 28–31) and obviously cannot discriminate past from persistent infections. A way to overcome these diagnostic shortcomings is opened by using a more standardized and nonsubjective test based on individual specific Ags of *C. pneumoniae.* Furthermore, the use of Ags that are differentially expressed during acute vs persistent infection might allow the diagnosis of persistently infected individuals which is an important prerequisite for clinical studies investigating the role of *C. pneumoniae* in chronic infections.

Although the *C. pneumoniae*-specific Ab response has been characterized by immunoblotting (32–35), only a few major Ags

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[4] Abbreviations used in this paper: MIF, microimmunofluorescence; EB, elementary body; 2-D, two dimensional; IPG, immobilized pH gradient; FT-ICR, Fourier transform ion cyclotron resonance; RT, room temperature; pI, isoelectric point; Pmp, polymorphic membrane protein.

Table I. *Primer sequences for PCR amplification*

| ORF (protein) | Primer Sequences[a] | Restriction Sites | Gene Region |
|---|---|---|---|
| CpB0579 (OMP2) | fw: CCGTCGACTGACTAAGATCGTCGCTAGTGCG | *Sal*I | 106–126 |
| | rv: CGCAAGCTTCACGTGGGTATTTTCTGTGTCT | *Hin*dIII | 1644–1665 |
| CpB0652 (RpoA) | fw: CCGGATCCGGGATGTCAGATAACGCACA | *Bam*HI | 0–17 |
| | rv: CCGTCGACATCTTTTCGGCATACCACTTC | *Sal*I | 1079–1100 |
| CpB0704 | fw: CCGGATCCGGAGCTCAAGGCGATAGCTC | *Bam*HI | 52–71 |
| | rv: CCGTCGACGATTAAAACTGTCATTGTATTCGC | *Sal*I | 1108–1131 |
| CpB0883 (PorB) | fw: CCGGATCCGCCCTAGGGGCTGGAAAC | *Bam*HI | 75–93 |
| | rv: CCGTCGACACTGCAGACCTGACGTAATG | *Sal*I | 998–1018 |
| CpB1000 (Pmp21-m) | fw: CCGGATCCGCTTGTAGTCATGGAGATCAT | *Bam*HI | 1908–1929 |
| | rv: CCGTCGACTGATATCTGCAAGTACTGGAG | *Sal*I | 3406–3427 |
| CpB1054 (Cpaf-c) | fw: CCGTCGACTGAGTGGTTTGATCCGTTCGACA | *Sal*I | 586–606 |
| | rv: CGCAAGCTTCGAAGCAGAAGTCGTTGTGG | *Hin*dIII | 1837–1857 |

[a] The primer-specific restriction enzyme sites are underlined; fw: forward; rv: reverse.

(i.e., MOMP, OMP2, and CrpA) have been identified thus far (36–38). The antigenic properties of MOMP that are controversial anyway (32–34) were shown to be conformation dependent (35, 37), hampering its use for serodiagnosis. Furthermore, *C. pneumoniae* OMP2 and CrpA, which were found to be highly immunogenic, displayed strong cross-reactivity with *Chlamydia trachomatis*-positive sera (38). Taken together, very little information is available regarding *C. pneumoniae* Ags that could be of use for serodiagnosis.

We have chosen a proteomic approach, combined with immunoblotting, to analyze the Ab-response pattern of different donors whose *C. pneumoniae* infection status was defined by MIF assay and by the detection of *C. pneumoniae* DNA by PCR. This work, in which numerous new *C. pneumoniae* Ags were identified, also provides new evidence for a characteristic Ab-response pattern found in donors persistently infected with *C. pneumoniae*.

## Materials and Methods

### *Human serum samples*

The prevalence of IgG Abs against *C. pneumoniae*, *C. trachomatis*, and *Chlamydia psittaci* in human sera was determined by MIF (Savyon Diagnostics). The MIF applied was based on whole EB of the same *C. pneumoniae* strain (i.e., TW183) used for the immunoblot analysis. Sera were collected from 39 human donors. From these, 27 sera (collective 1) were obtained from healthy donors at the University of Konstanz (Konstanz, Germany) whose *C. pneumoniae* serostatus had previously been assessed by MIF. The sera were randomly chosen to generate three groups of nine sera each with MIF titers of <64, 64–256, or ≥512. Twelve sera (collective 2) originated from a clinical study, which included patients who underwent heart transplantation (7). One serum of the second collective was excluded in our study, because it tested positive for *C. trachomatis* Abs. As for the 12 heart transplant patients, none of the 27 healthy donors had a clinical history of recent infections of the respiratory tract.

### *Detection of* C. pneumoniae *by PCR*

PBMC samples of 23 donors of collective 1 were taken at four different time points during a period of 18 mo and were analyzed at each time point for the presence of *C. pneumoniae* DNA by PCR by blinded investigators. For this purpose, $5 \times 10^6$ PBMC were collected using Vacutainer CPT (BD Biosciences) and DNA was prepared and resuspended in 100 $\mu$l of water. For *C. pneumoniae* detection, 25 $\mu$l of extracted DNA (DNA amount ranged from 0.5 to 1.0 $\mu$g) was subjected to PCR using the HL-1/HR-1 primers that amplify a genomic 438-bp *C. pneumoniae* target sequence. For enhanced specificity, a nested PCR protocol was used with the nested oligonucleotide primer pair In-1 (5′-AGT TGA GCA TAT TCG TGA GG-3′) and In-2 (5′-TTT ATT TCC GTG TCG TCC AG-3′), which yield a 128-bp product (39). For confirmation, nonradioactive DNA hybridization was performed using oligonucleotide HM-1 3′-tailed with digoxigenin-11-dUTP/dATP (Roche Diagnostics) as probe. PCR was controlled according to current guidelines (26).

### *Cultivation and preparation of* C. pneumoniae

*C. pneumoniae* TW183 (a gift of Prof. J. Kuipers, Department of Rheumatology, Medical School of Hannover, Hannover, Germany) were propagated in HEp-2 cells (CCL-23; American Type Culture Collection), maintained in MEM medium supplemented with 10% heat-inactivated FCS, 1 mM sodium pyruvate, and 1% nonessential amino acids (PAA) at 37°C in a 5% $CO_2$ atmosphere. Semiconfluent monolayers of cells in 6-well trays were infected with 5–10 inclusion forming units/cell of *C. pneumoniae* TW183 × 50 min of centrifugation at 1,300 × *g*. Infected cells were cultivated in MEM supplemented with 5% heat-inactivated FCS, 1% nonessential amino acids, 0.45% glucose (Sigma-Aldrich), and 2 $\mu$g/ml cycloheximide. After 72 h, infected cells were scraped off with 2 ml/tray HBSS (PAA) with $Ca^{2+}/Mg^{2+}$ containing 5 U/ml heparin and 0.1 mg/ml DNase I (Roche), as well as protease inhibitor (P8340; Sigma-Aldrich). Cells were disrupted by vortexing with glass beads and centrifuged for 10 min at 1,500 × *g* to sediment cellular debris. The supernatant was incubated for 20 min at 37°C, transferred to 38-ml centrifuge tubes (Nalge Nunc International) discontinuous gradient containing 25/50% gastrografin (Schering), and centrifuged at 45,000× *g* for 60 min at 8°C. *C. pneumoniae*

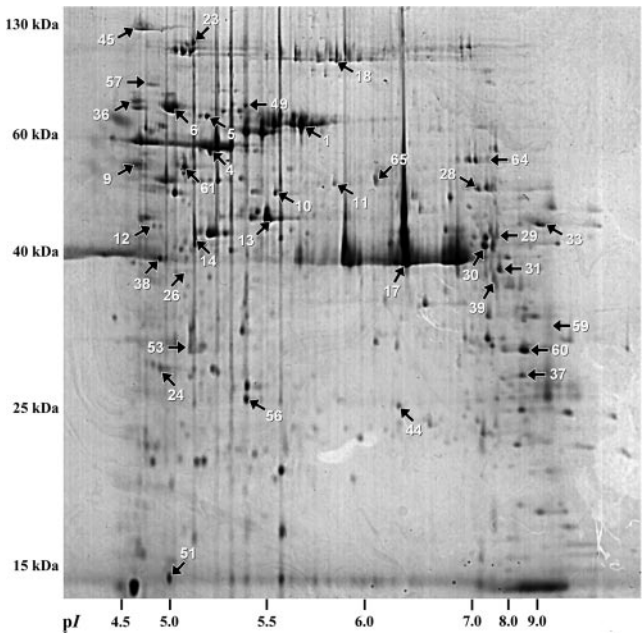


**FIGURE 1.** Total protein extract from *C. pneumoniae* TW183 was separated by 2-D gel electrophoresis and stained with colloidal Coomassie. All *C. pneumoniae* protein spots marked with a number where identified by mass spectrometry analysis (see Tables II and III). The spots 29, 49, 60, 61, and 64 represent human proteins deriving from HEp-2 host cells used for the propagation of *C. pneumoniae*.

Table II. *Identification and characterization of* C. pneumoniae *Ags*

| Functional Class | Spot No. | Protein: *C. pneumoniae* TW183 (synonym) | ORF: CpB | Theoretical pI/$M_r$[a] | Observed pI/$M_r$ | % Sequence Coverage | Freq.[b]: $n = 38$ | React.[c]: (%) |
|---|---|---|---|---|---|---|---|---|
| Cell envelope | 1 | 60-K cysteine-rich OMP (OMP2) | 0579 | 5.73/59.7 | 5.68/62.9 | 52 | 27 | 25.6 |
| | 38 | Major outer membrane protein ($MOMP_{4.9}$)[d] | 0722 | 6.13/39.3 | 4.94/39.2 | 11 | 10 | 9.4 |
| | 17 | Major outer membrane protein ($MOMP_{6.2}$)[d] | 0722 | 6.13/39.3 | 6.17/38.6 | 29 | 13 | 9.0 |
| | 53 | Outer membrane protein B (PorB)[e] | 0883 | 5.10/34.4 | 5.12/30.1 | 42 | 7 | 5.3 |
| Pmp | 18 | Outer membrane protein 7 (Pmp2)[e] | 0015 | 5.33/73.0 | 5.85/88.6 | 36 | 7 | 5.6 |
| | 45 | Outer membrane protein 11 (Pmp6)[e] | 0460 | 5.28/142.3 | 4.75/130.5 | 21 | 23 | 18.0 |
| | 23 | Outer membrane protein 5 (Pmp10)[e] | 0467 | 5.22/94.4 | 5.10/97.0 | 37 | 12 | 8.3 |
| | 36 | Outer membrane protein 11 (Pmp21-n)[f] | 1000 | 4.84/168.4 | 4.67/70.6 | 8 | 6 | 3.8 |
| | 9 | Outer membrane protein 11 (Pmp21-m)[e,f] | 1000 | 4.84/168.4 | 4.68/54.6 | 21 | 13 | 9.4 |
| | 10 | Outer membrane protein 11 (Pmp21-c)[e,f] | 1000 | 4.84/168.4 | 5.55/49.2 | 16 | 10 | 8.3 |
| Transport related | 12 | Low calcium response E (LcrE)[e] | 0334 | 4.98/43.4 | 4.87/43.6 | 44 | 10 | 9.4 |
| | 24 | Type III translocation protein L (YscL)[e] | 0855 | 4.96/25.8 | 4.96/28.7 | 45 | 9 | 4.9 |
| | 57 | Outer membrane secretion protein (YscC)[e] | 0729 | 6.02/100.4 | 4.85/77.7 | 24 | 7 | 4.9 |
| Chaperones | 4 | Chaperonin Hsp60 (GroEL) | 0135 | 5.29/58.2 | 5.25/58.0 | 21 | 23 | 19.5 |
| | 6 | Molecular chaperone Hsp70 (DnaK) | 0523 | 4.99/71.4 | 5.02/67.6 | 17 | 15 | 11.3 |
| Transcription | 28 | Transcription termination factor (Rho) | 0634 | 6.80/51.8 | 7.09/50.1 | 25 | 7 | 4.9 |
| | 14 | RNA polymerase $\alpha$-chain (RpoA) | 0652 | 5.07/41.9 | 5.13/41.0 | 36 | 13 | 10.9 |
| Translation | 13 | Elongation factor Tu (EF-Tu) | 0074 | 5.43/43.1 | 5.51/44.7 | 39 | 28 | 25.2 |
| | 51 | Ribosomal protein L7/L12 (RplL)[g] | 0080 | 5.02/13.6 | 5.00/13.5 | 29 | 3 | 4.9 |
| | 5 | Ribosomal protein S1 (RpsA) | 0325 | 5.16/65.0 | 5.20/65.0 | 7 | 7 | 4.9 |
| | 59 | Ribosomal protein S2 (Rs2)[e] | 0723 | 8.70/31.3 | 9.36/32.0 | 48 | 3 | 2.3 |
| Energy | 39 | Fructose bisphosphate aldolase (DhnA) | 0289 | 7.16/38.0 | 7.55/37.0 | 21 | 5 | 2.3 |
| Metabolism | 11 | Dihydrolipoamide acetyltransferase (PdhC)[e] | 0315 | 5.90/46.3 | 5.84/51.0 | 41 | 3 | 3.4 |
| Mixed | 65 | Leucyl aminopeptidase (PepA)[e] | 0397 | 5.95/54.5 | 6.04/51.6 | 58 | 3 | 2.6 |
| | 56 | Uridylate kinase (PyrH) | 0725 | 5.39/27.1 | 5.40/25.7 | 29 | 3 | 2.3 |
| | 31 | Recombinase A (RecA) | 0790 | 7.71/38.2 | 7.74/38.0 | 28 | 7 | 6.0 |
| | 44 | CPAF (Cpaf-n)[h] | 1054 | 5.32/67.1 | 6.14/25.1 | 16 | 4 | 2.3 |
| | 26 | CPAF (Cpaf-c)[e,h] | 1054 | 5.32/67.1 | 5.09/38.0 | 22 | 7 | 7.9 |
| Hypothetical | 37 | Hypothetical protein (CpB0546)[e] | 0546 | 8.57/29.6 | 8.55/28.0 | 70 | 10 | 7.1 |
| | 30 | Hypothetical protein (CpB0704) | 0704 | 7.16/40.6 | 7.20/40.6 | 36 | 27 | 30.1 |
| | 33 | Hypothetical protein (CpB0837)[e] | 0837 | 9.38/44.5 | 9.09/44.0 | 34 | 18 | 13.9 |

[a] The theoretical pI and $M_r$ values were calculated using the Compute pI/Mw tool at the ExPASy server (leader peptides predicted with SignalP were cleaved off before calculation).
[b] Frequency (Freq.) indicates the number of reacting sera.
[c] Reactivity (React.) was calculated as the sum of the intensity classes (0–7) observed among the 38 sera and given in percent of the maximal reachable of value ($38 \times 7$).
[d] Two MOMP isoforms (pI 4.9, pI 6.2) exhibiting different serum reactivity were identified.
[e] Proteins identified by ProteoSys.
[f] Three different fragments of OMP11 were identified corresponding to the N-terminal (Pmp21-n), middle (Pmp21-m), and C-terminal part (Pmp21-c) of the full-length protein.
[g] RplL was not resolved in all gels resulting in underestimated reactivity.
[h] Two different fragments of CPAF were identified corresponding to the N-terminal (Cpaf-n) and C-terminal part (Cpaf-c) of the full-length protein.

EB were collected from the 25/50% interphase and washed twice with HBSS followed by centrifugation for 30 min at 30,000× *g*. The pellet was resuspended by sonication in 1% SDS, 50 mM Tris-HCl (pH 7), then boiled for 5 min, and stored in aliquots at −80°C. The protein concentration of the samples was determined using the Uptima bicinchoninic acid assay (Interchim). Before two-dimensional (2-D) electrophoresis, the proteins of the samples were purified using 2-D Clean-up kit (Amersham Biosciences) according to the manufacturer's instructions.

### *2-D gel electrophoresis*

For 2-D gel electrophoresis, a sample containing 550 $\mu$g of *C. pneumoniae* protein was mixed with immobilized pH gradient (IPG) rehydration buffer containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 40 mM Tris base, 2% v/v Servalyt (pH 3–10; Serva), 0.3% w/v DTT, and a trace of bromphenol blue and applied overnight to 17-cm IPG strips (pH 3–10 nonlinear; Bio-Rad). Isoelectric focusing was performed at 20°C for 1 h at 150 V, 0.5 h at 150–300 V, 2 h at 300 V, 2.5 h at 300–3500 V, and 6 h at 3500 V using a Multiphor II (Amersham Biosciences). After the first dimension, the IPG strips were equilibrated in 6 M urea, 50 mM Tris-HCl (pH 8.8), 30% v/v glycerol, 2% w/v SDS, a trace of bromphenol blue, and 1% w/v DTT for 40 min. In a second equilibration step, DTT was replaced by 4.5% w/v iodoacetamide for 20 min. 2-D electrophoresis was conducted on a Protean II xi system (Bio-Rad) using SDS-polyacrylamide gels (10% polyacrylamide). Protein separation was performed at 25 mA per gel for ~30 min and 40 mA per gel until the dye front reached the anodic end of the gels. The gels were stained with colloidal Coomassie (Sigma-Aldrich) according to Neuhoff (40) and scanned using a GS-710 calibrated imaging densitometer (Bio-Rad).

### *Immunoblotting*

For immunoblot analysis, 2-D gels were electroblotted onto nitrocellulose membranes (Pall) for 2 h at 60 V using a WEB-M tank blotter (PeqLab) with a buffer containing 25 mM Tris, 192 mM glycine, and 10% methanol. Next, the membranes were blocked with 5% nonfat dry milk in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20 (pH 7.4)) for 2 h at room temperature (RT) and incubated with human sera at a dilution of 1/1000 overnight at 4°C. The membranes were washed with TBST four times for 15 min and incubated with a peroxidase-conjugated rabbit anti-human IgG secondary Ab (1/2000 diluted; DakoCytomation) for 45 min at RT. After four subsequent washing steps, immunoreactive spots were visualized by ECL detection by 5-min exposure to a LAS-3000 imaging system (Fuji) using following settings: VRF43LMD lens, iris = 0.85, standard sensitivity/resolution, 1536 × 1024 pixel (pixel size 144 × 144 $\mu$m). After detection of immunoreactive spots, the membranes were washed and abundant protein spots were stained with 0.2% Ponceau S (Sigma-Aldrich) in 5% acetic acid.

### *Evaluation of the immunoblots*

The electronic image files of the blots were analyzed and matched with the 2-D electrophoretic map of the respective Coomassie-stained gels. In addition to the immunoreactive spots on the membranes, the location of 20 Ponceau-stained proteins was used to optimize the alignment. For the analysis of the immunoblots, the maximum intensity of each spot was determined (0–64,000 events) using the AIDA software package (Raytest/Fuji). Thereby, an intensity threshold of 500 events above background level was used as cutoff and all spots exhibiting a higher intensity were considered to

Table III. *Identification and characterization of proteins with human origin*

| Spot No. | Proteins with Human Origin | Gene ID | Theoretical pI/$M_r$[a] | Observed pI/$M_r$ | % Sequence Coverage | Freq.[b] $n = 38$ | React.[c] (%) |
|---|---|---|---|---|---|---|---|
| 49 | Heat shock 70-kDa protein 9 (MTHSP75)[d] | 3313 | 5.87/73.7 | 5.40/69.1 | 39 | 3 | 2.3 |
| 61 | Tubulin $\alpha$-4A chain | 7277 | 4.95/49.9 | 5.08/54.2 | 31 | 3 | 3.0 |
| 29 | UQCRC2[d] | 7385 | 8.74/48.4 | 7.41/42.9 | 41 | 5 | 6.4 |
| 60 | Porin 31HM[d] | 7416 | 8.62/30.8 | 8.63/30.2 | 80 | 4 | 2.3 |
| 64 | Chaperonin containing TCP1, subunit 7[d] | 10574 | 7.55/59.4 | 7.27/55.6 | 26 | 3 | 4.1 |

[a] The theoretical pI and $M_r$ values were calculated using the Compute pI/Mw tool at the ExPASy server (leader peptides predicted with SignalP were cleaved off before calculation).
[b] Frequency (Freq.) indicates the number of reacting sera.
[c] Reactivity (React.) was calculated as the sum of the intensity classes (0–7) observed among the 38 sera and given in percent of the maximal reachable of value ($38 \times 7$).
[d] Proteins identified by ProteoSys.

be immunoreactive spots. To enable a comparative analysis, the measured maximal intensities of the spots were classified into eight categories based on a $\log_2$ scale (0, <500; 1, <1,000; 2, <2,000; 3, <4,000; 4, <8,000; 5, <16,000; 6, <32,000; 7, <64,000 events/pixel). To compare the frequency and the intensity of immunoreactive spots, the overall reactivity was calculated in percent of the maximal reachable value for each spot. For this purpose, the sum of the measured intensity categories for a number of donors ($n$) was divided by the product of ($n$) $\times$ (the highest achievable intensity of 7) and multiplied with 100%. Only spots that achieved a reactivity of ≥2% were analyzed by peptide mass fingerprinting.

### *Peptide mass fingerprinting*

For high-resolution MALDI-Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry, protein spots from *C. pneumoniae* EB were excised from Coomassie-stained gels, washed with water, and with acetonitrile/water (3:2) for 15 and 30 min, respectively, and dried in a vacuum centrifuge. In-gel digestion and extraction of peptides were performed according to Mortz et al. (41). The samples were desalted with C18 OMIX Pipette Tips (Varian) and eluted with a solution containing 50% acetonitrile and 0.1% trifluoroacetic acid. A 1-$\mu$l sample was mixed with 0.5 $\mu$l of matrix solution containing 100 mg/ml 2,5-dihydroxybenzoic acid in acetonitrile/water with 0.1% trifluoroacetic acid (2:1) and applied to a stainless steel MALDI target. MALDI-FT-ICR mass spectrometric analysis was performed with a Bruker APEX II FT-ICR instrument. Acquisition and processing of spectra were performed with XMASS software (Bruker). With the resulting monoisotopic peptide masses, database searches were performed using MASCOT search engine (www.matrixscience.com) as well as the ProFound search engine (http://prowl.rockefeller.edu/) with restriction to mass tolerances of maximal 10 ppm. Some protein samples were analyzed by ProteoSys by MALDI-TOF peptide mass fingerprinting, as published previously (42).

### *Recombinant expression of* C. pneumoniae *proteins*

Genomic DNA from gradient-purified EB of *C. pneumoniae* TW183 was prepared using the DNeasy tissue kit (Qiagen). The full-length open reading frames without signal sequences of Omp2, RpoA, CpB0704, PorB, the middle part of Pmp21, and the C-terminal part of Cpaf were cloned into the pQE30 expression vector (Qiagen) that encodes a hexahistidine tag. Candidate genes were amplified by PCR from 9 ng of genomic DNA using Pfu Polymerase (Fermentas) and primers were flanked with specific restriction sites (Table I). The PCR products were purified from agarose gels, ligated into the pQE30 vector, and used for the transformation of XL2-Blue ultracompetent *Escherichia coli* cells (Stratagene). Protein expression was induced with 1 mM isopropyl $\beta$-D-thiogalactoside in transformed *E. coli* (strain M15) cells (Qiagen) for 6 h at 30°C. The cells were harvested by centrifugation and disrupted by resuspension in Ni-NTA-binding buffer (20 mM phosphate buffer (pH 7.5), 30 mM imidazole, 500 mM NaCl, and 8 M urea) for 1 h at 4°C. Then, 1-ml HisTrap HP columns (Amersham Biosciences) were loaded with the supernatant of the cells, washed with 20 volumes of Ni-NTA-binding buffer, and eluted with a buffer containing 500 mM imidazole. The eluted fractions were dialyzed with PBS using Amicon Ultra centrifugal filter (Millipore) and analyzed by SDS-PAGE. The concentration of the purified proteins was determined using the bicinchoninic acid assay (see above).

### *ELISA*

ELISAs for the determination of human rOmp2, rRpoA, rCpB0704, rPorB, rPmp21-m, and rCpaf-c Abs were established and optimized. In brief, the wells of a microtiter plate (Nunc) were coated with 200 ng of recombinant protein in 0.1 M sodium carbonate buffer (pH 8.2) overnight at 4°C. After being washed with PBST (10 mM sodium phosphate, 140 mM NaCl, 0.1% Tween 20 (pH 7.0)), the wells were blocked with 5% nonfat dry milk in PBST for 2 h. Human serum samples were diluted 1/200 in blocking solution and 100 $\mu$l were added to the wells and incubated for 2 h at RT. After three washing steps, the wells were incubated with 100 $\mu$l of peroxidase-conjugated rabbit anti-human IgG secondary Ab (Dako-Cytomation), diluted 1/2000 in blocking solution, for 30 min at RT. The substrate (3,3′,5,5′-tetramethylbenzidine; Sigma-Aldrich) was added after six washing steps. The reaction was stopped with 50 $\mu$l of $H_2SO_4$, and the OD was measured at 450 nm.

### *Statistics*

Statistical analysis was performed using GraphPad Prism 4 software. To compare the frequency and the intensity of spot between three groups of donors, the Kruskal-Wallis test followed by Dunn test was used; *, **, *** indicate a significant difference between particular groups with $p \leq 0.05$, 0.01, and 0.001, respectively.

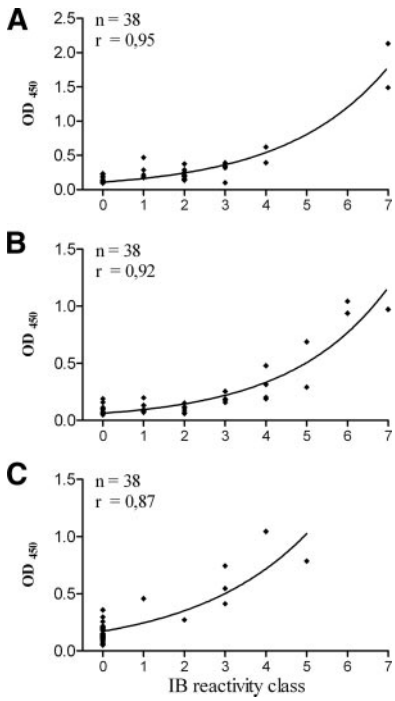


**FIGURE 2.** The reactivity of *C. pneumoniae* proteins in ELISA experiments was compared with that observed in the immunoblots. Recombinantly expressed OMP2 (*A*), CpB0704 (*B*), and Cpaf-c (*C*) were immobilized to ELISA wells and incubated with each of 38 human sera. Then, the proportion of bound human IgG Abs was determined. The correlation between the ELISA values ($OD_{450}$) and the immunoblot (IB) reactivity class was tested using a nonlinear regression analysis.

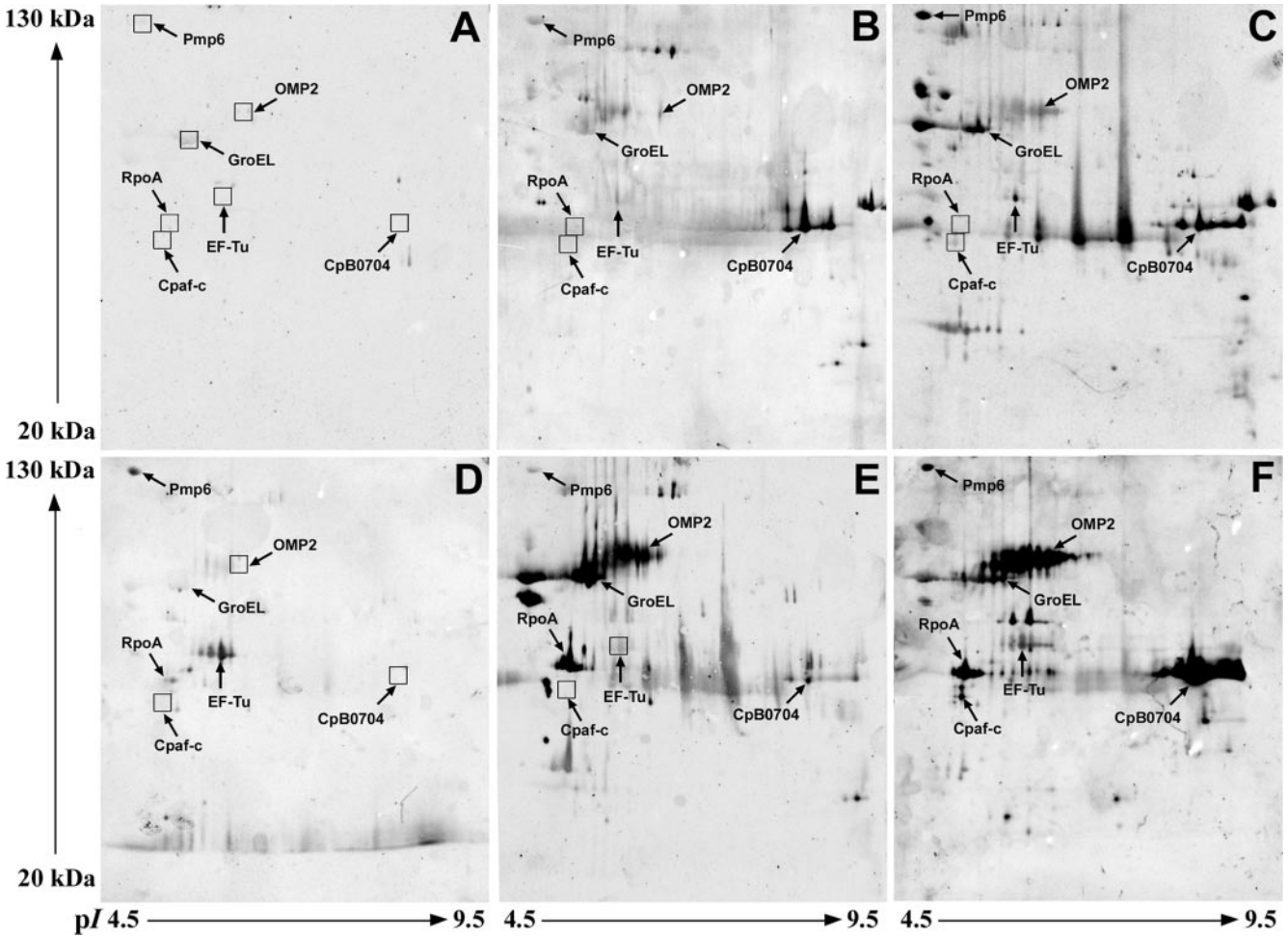


**FIGURE 3.** Immunoblots were prepared with sera from six different donors and analyzed using a LAS-3000 imaging system. *C. pneumoniae* TW183 proteins separated by 2-D gel electrophoresis were used for immunoblotting. The blots were incubated with sera from two MIF-negative donors (titer <64) (*A* and *D*), two MIF-positive donors (titer 64 and 128, respectively) (*B* and *E*), as well as with two sera from highly MIF-positive donors (titer ≥512) (*C* and *F*). The PBMC of donors D–F were tested as positive for *C. pneumoniae* DNA by PCR. The arrows indicate selected Ags that have been identified by mass spectrometry analysis (results given in Tables II and III). The boxed regions indicate the respective spots with luminescence intensities lower than the cutoff.

## Results

### *2-D gel electrophoresis and immunoblot analysis*

To identify and analyze *C. pneumoniae* Ags, 2-D gel electrophoresis was performed in combination with immunoblotting and peptide mass fingerprinting. The 2-D gels were prepared in parallel under identical conditions using equal amounts of proteins extracted from the same batch of *C. pneumoniae* EB. In each run, three 2-D gels were used for immunoblotting and one gel was stained with colloidal Coomassie. In Fig. 1, a representative 2-D gel of separated *C. pneumoniae* proteins is shown. Using nonlinear pH 3–pH 10 IPG strips, ~500–600 protein spots were resolved in a molecular mass range from 15 to 130 kDa, which is in agreement with other studies (43, 44). Immunoblots were prepared using 38 sera with different *C. pneumoniae* Ab titers, which were negative for *C. trachomatis* and *C. psittaci* Abs by MIF. The reproducibility of the blots was confirmed in independent experiments. For the blot evaluation, the maximal luminescence intensity of each spot was determined. In case of a spot series, the maximal intensity of the main spot was determined. Using an intensity cutoff for immunoreactive spots of 500 events per pixel, a total of 75 reactive spots or spot series were detected among the 38 sera tested. To enable comparative analysis, the reactivity of each spot corresponding to the frequency and intensity of recognition by different sera was calculated in percent of the maximal reachable value (for explanation, see Tables II and III). The evaluation of the 38 immunoblots revealed 42 spots that exhibited a reactivity of >2%.

### *Identification of* C. pneumoniae *Ags*

To identify the most immunogenic proteins of *C. pneumoniae*, the 42 spots exhibiting the strongest reactivity were excised from the gels and used for mass spectrometry analysis after in-gel digestion with trypsin. The determination of the tryptic peptide masses of the 42 main spots revealed 31 different *C. pneumoniae* protein species originating from 27 *C. pneumoniae* genes (Table II, see also Fig. 1). A great number of the identified spots represented cell envelope associated and polymorphic membrane proteins (Pmp), such as MOMP, OMP2, PorB, Pmp2, Pmp6, Pmp10, and Pmp21. Furthermore, a variety of proteins involved in different cellular processes, e.g., transport, transcription, and translation, were identified. Among the 38 sera, the highest reactivity (≥10%) was observed for OMP2, Pmp6, GroEL, DnaK, RpoA, EF-Tu, CpB0704, and CpB0837.

For Pmp21 and Cpaf, three and two different parts of the full-length proteins, respectively, but not the full-length protein itself, were identified, which is in agreement with data from other studies (45, 46). In the case of MOMP, two isoforms of the full-length protein were identified (isoelectric point (pI) 4.9 and 6.2) that showed different antigenic properties. Among the 42 immunoreactive spots analyzed, 6 spots turned out to be fragments of the identified full-length proteins DnaK, EF-Tu, MOMP, and Pmp6 (data not shown). In addition, 5 spots were identified as human-derived proteins, such as mitochondrial proteins or tubulin $\alpha$, which likely represent contaminations from the HEp-2 cells used for the propagation of *C. pneumoniae* (Table III).

### *Confirmation of the immunoblot results using rAgs*

To determine the validity and the sensitivity of the 2-D immunoblot approach, the serum reactivity toward three identified proteins was analyzed by ELISA and compared with the immunoblot results. For this purpose, the full-length proteins of OMP2 and CpB0704 as well as the C-terminal part of Cpaf were expressed recombinantly and immobilized on ELISA wells. Then, each of the 38 sera used for immunoblotting was incubated with the proteins and the proportion of bound human IgG Abs was analyzed. As shown in Fig. 2, for all three candidate proteins the observed ELISA values showed a high correlation with the intensities determined by immunoblotting ($r = 0.95$, $r = 0.92$, and $r = 0.87$, respectively).

### *Identification of Ags possibly contributing to MIF reactivity*

In all sera used for the immunoblots, the prevalence of *C. pneumoniae* Abs was analyzed by MIF. Twenty-seven of 38 sera were tested positive by MIF ($n = 17$, titer 64–256 and $n = 10$, titer ≥512), whereas 11 sera had very low or no detectable Abs ($n = 11$, titer <64). As indicated in Fig. 3, the number of reactive spots and the intensity of recognition were associated with the Ab titer of the sera as determined by MIF. Although sera with MIF titer ≥512 reacted with 10–20 (mean 14.1) of the 31 identified protein species, sera with titers 64–256 and a titer <64 reacted with 3–14 (mean 9.4) and 1–10 (mean 3.5), respectively. To identify the Ags that possibly contribute to the MIF reactivity, the reactivity of the proteins achieved by each of the three different groups of sera (MIF <64; 64–256; ≥ 512) was determined and compared using statistical analysis. Thereby, we identified 14 individual proteins exhibiting significantly higher reactivity with sera having a MIF titer of 64–256 or ≥512, respectively, compared with MIF-negative sera (Table IV).

Table IV. *Identification of MIF-associated Ag*[a]

| | Titer < 64 | | Titer 64–256 | | Titer ≥ 512 | |
|---|---|---|---|---|---|---|
| | ($n = 11$) | | ($n = 17$) | | ($n = 10$) | |
| Protein | Freq. | React. | Freq. | React.[b] | Freq. | React.[b] |
| CpB0704 | 2 | 4 | 16 | 30* | 9 | 59** |
| Cpaf-c[c] | 0 | 0 | 0 | 0 | 7 | 30** |
| Pmp10 | 0 | 0 | 4 | 7 | 8 | 20** |
| CpB0837 | 0 | 0 | 10 | 16*** | 8 | 26** |
| GroEL | 2 | 3 | 13 | 22* | 8 | 34* |
| OMP2 | 3 | 8 | 14 | 29*** | 10 | 39* |
| Pmp6 | 3 | 6 | 11 | 15 | 9 | 36* |
| PorB | 0 | 0 | 2 | 4 | 5 | 13* |
| $MOMP_{4.9}$[d] | 1 | 1 | 5 | 9 | 7 | 17* |
| Pmp21-c[c] | 0 | 0 | 5 | 7 | 5 | 20*** |
| YscL | 0 | 0 | 4 | 5 | 5 | 10*** |
| CpB0546 | 0 | 0 | 5 | 8 | 5 | 13*** |
| $MOMP_{6.2}$[d] | 0 | 0 | 5 | 14 | 5 | 11*** |
| LcrE | 0 | 0 | 7 | 13*** | 3 | 14 |

[a] Identification of *C. pneumoniae* Ags that exhibited significant higher reactivity with sera from donors having *C. pneumoniae* IgG titer (determined by MIF assay) of 64–256 or ≥512 compared to donors with an IgG titer <64. Frequency (Freq.) indicates the number of reacting sera and reactivity (React.) was calculated as the sum of intensity classes (0–7) for each of the three donor groups, respectively, and given in percent of the maximal reachable value.

[b] *, **, and *** indicate significant differences with $p < 0.01$, 0.001, and 0.05, respectively.

[c] Cpaf-c and Pmp21-c represent C-terminal parts of the full-length proteins.

[d] $MOMP_{4.9}$ and $MOMP_{6.2}$ represent different isoforms (pI 4.9, pI 6.2) of the protein.

### *Analysis of Ab-response pattern among donors with persistent* C. pneumoniae *infection*

A major drawback of current *C. pneumoniae* serodiagnosis is the inability to discriminate between past and persistent infections. In our experiments, we included sera from 11 heart transplant patients (collective 2) originating from a study of Ramirez et al. (7), who provided evidence for persisting *C. pneumoniae* in 5 of these patients by detecting *C. pneumoniae* DNA in their coronary arteries. Furthermore, we included sera of 27 healthy donors (collective 1), of whom we analyzed the presence of *C. pneumoniae* DNA in PBMC, which is also considered as a marker for persistent infections. Except in the case of four donors who could not be recruited for this analysis, PBMC samples from all healthy donors were collected at four different time points over a period of 18 mo. Among the 23 healthy donors analyzed, PBMC samples from 14 donors tested positive for *C. pneumoniae* DNA, whereas 9 donors were negative in all samples. As observed for the heart transplant patients, the presence of *C. pneumoniae* DNA in healthy donors did not correlate with MIF titer (Table V). For the determination of the Ab-response pattern of donors with evidence for persisting *C. pneumoniae* (PCR-positive donors), collectives 1 and 2 were combined, and the immunoblot reactivity of 15 PCR-negative and 19 PCR-positive donors was compared. Thereby, 12 individual *C. pneumoniae* proteins were identified whose reactivity with sera from PCR-positive donors was >1.5-fold different to that of PCR-negative donors (Table VI). Among these proteins, RpoA, MOMP (pI 4.9), YscC, Pmp10, PorB, Pmp21-m, GroEL, and Cpaf-c showed higher reactivity and YscL, Rho, LcrE, and CpB0837 showed lower reactivity with sera from PCR-positive donors. As indicated in Fig. 3, the observed differences were highest for RpoA and Cpaf-c. Consistent results were also obtained when collectives 1 and 2 were considered separately for the analysis.

To exemplarily verify the discriminative potential of these proteins by ELISA, the full-length proteins of RpoA, PorB, the middle

Table V. *C. pneumoniae-DNA status of healthy donors and heart transplant patients with respect to their* C. pneumoniae *serum IgG titer*[a]

| | *C. pneumoniae*-DNA status | | | |
|---|---|---|---|---|
| | Healthy Donors, $n = 23$ | | Transplant Patients, $n = 11$ | |
| MIF Titer | $PCR^+$ | $PCR^-$ | $PCR^+$ | $PCR^-$ |
| <64 | 5 | 2 | 1 | 1 |
| 64–256 | 3 | 6 | 3 | 5 |
| ≥512 | 6 | 1 | 1 | |

[a] The presence of *C. pneumoniae* DNA in PBMC samples of 23 healthy donors and coronary arteries of 11 heart transplant patients was analyzed using PCR and compared to the *C. pneumoniae* serum IgG titer of the donors as determined by MIF.

Table VI. *Immunoblot analysis dependent on the prevalence of* C. pneumoniae *DNA*[a]

| Protein | Frequency PCR$^-$ $n = 15$ | Frequency PCR$^+$ $n = 19$ | Reactivity PCR$^-$ $n = 15$ | Reactivity PCR$^+$ $n = 19$ | Reactivity[b] Fold Difference |
|---|---|---|---|---|---|
| Cpaf-c[c] | 0 | 6 | 0.0 | 14.3 | >1.5 ↑ |
| RpoA | 2 | 10 | 2.8 | 18.1 | 6.3 ↑ |
| $MOMP_{4.9}$[d] | 2 | 6 | 3.8 | 13.5 | 3.6 ↑ |
| YscC | 1 | 4 | 1.9 | 6.77 | 3.6 ↑ |
| Pmp10 | 3 | 7 | 3.8 | 9.8 | 2.6 ↑ |
| PorB | 1 | 5 | 2.9 | 7.5 | 2.6 ↑ |
| Pmp21-m[e] | 5 | 7 | 5.7 | 12.0 | 2.1 ↑ |
| GroEL | 9 | 12 | 15.2 | 24.1 | 1.6 ↑ |
| YscL | 5 | 2 | 6.7 | 2.3 | 2.7 ↓ |
| Rho | 4 | 2 | 7.6 | 3.0 | 2.5 ↓ |
| LcrE | 6 | 3 | 12.4 | 6.0 | 2.1 ↓ |
| CpB0837 | 9 | 7 | 19.1 | 10.5 | 1.8 ↓ |

[a] The frequency and reactivity of the 23 most frequent *C. pneumoniae* Ags was analyzed with respect to the *C. pneumoniae*-DNA status of the donors. Frequency indicates the number of reacting sera and reactivity was calculated as the sum of intensity classes (0–7) for each of the two donor groups (PCR$^+$/PCR$^-$), respectively, and given in percent of the maximal reachable value.

[b] (↑) and (↓) indicate higher and lower reactivity with sera from PCR$^+$ donors, respectively.

[c] Cpaf-c represents the C-terminal part of the full-length protein.

[d] $MOMP_{4.9}$ represents one of the two identified MOMP isoforms (pI 4.9, pI 6.2).

[e] Pmp21-m represents the middle part of the full-length protein.

part of Pmp21, and the C-terminal part of Cpaf were recombinantly expressed and used to determine the serum reactivity of the donors used for immunoblotting. Among the sera of 19 PCR-positive donors, 6, 7, 3, and 8 sera reacted with RpoA, PorB, Pmp21-m, and Cpaf-c, respectively, while no positive reaction was observed with the sera of 15 PCR-negative donors (Fig. 4). Together, from the 19 sera of PCR-positive donors, 5 sera reacted with one and 9 sera reacted with two or three of the four proteins. Although the ELISA experiments confirmed the results of the immunoblots, for RpoA and Pmp21-m the sensitivity of the ELISA was lower compared with the immunoblots.

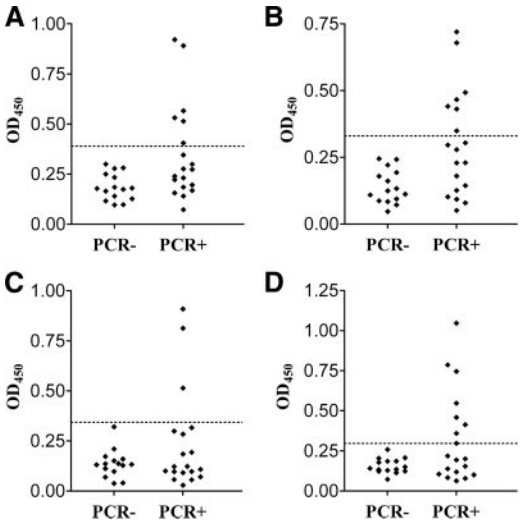


**FIGURE 4.** The discriminative seroreactivity of *C. pneumoniae* proteins was verified by ELISA experiments. Recombinantly expressed RpoA (*A*), PorB (*B*), and Pmp21-m (*C*) and Cpaf-c (*D*) were immobilized to ELISA wells and incubated with each of 15 sera of PCR-negative and 19 sera of PCR-positive donors. Then, the proportion of bound human IgG Abs was measured. The cutoff for a positive reaction (indicated as a dotted line) was calculated from the mean $OD_{450}$ of PCR-negative donors plus three SDs.

## Discussion

Serologic tests allowing differential diagnosis of the *C. pneumoniae* infection status, like past vs persistent infection, represent a possible key to solve the controversy about the role of *C. pneumoniae* in chronic inflammatory diseases. The identification of specific *C. pneumoniae* Ags in general as well as of those associated with the status of persistent infection are prerequisites for the development of such tests. For our approach, we used proteomics, combined with 2-D gel immunoblotting, to analyze IgG Ab-response pattern of different sera. Altogether, by mass spectrometry analysis, we have identified 31 major *C. pneumoniae* Ags, of which 12 were found to be characteristic either for persistent or nonpersistent infection of the donors.

As expected, we identified several *C. pneumoniae* proteins with known antigenic properties, such as MOMP, OMP2, PorB, Pmp10, and GroEL (37, 38, 47–49), as well as proteins that were shown to be antigenic in *C. trachomatis*, such as CPAF, Pmp21, LcrE, DnaK, EF-Tu, RpsA, RpoA, and RplL (50–52). More than one-half of the proteins are described here for the first time to be antigenic, including some with high antigenic potential, such as Pmp6 and the hypothetical proteins CpB0704 and CpB0837. The latter three exhibited, together with OMP2, GroEL, DnaK, RpoA, and EF-Tu, the highest immunoblot reactivity, indicating that these proteins represent dominant Ags of *C. pneumoniae*. Interestingly, in our proteomic approach the chlamydial protease Cpaf was identified, although it has been demonstrated to be typically absent from purified EB preparations, but present in the lysate of infected cells (45). We assume that despite its secretion into the host cell, traces of this protein remain in the EB and that the detection of Cpaf in our study results from using large amounts of EB proteins for the 2-D electrophoresis. The detection of Cpaf in our study might also be due to using a different *C. pneumoniae* strain (TW 183) that probably has a higher expression level or a lower secretion capacity for Cpaf. In agreement with other studies that used immunoblotting, *C. pneumoniae* MOMP was found not to be a dominant Ag (32, 37). Although some investigators observed a highly immunoreactive band at the molecular mass of MOMP (33, 34), we speculate that this band was rather caused by the hypothetical protein CpB0704, which has almost the same molecular mass and exhibited the highest immunoblot reactivity in our study. In the 2-D gels, we consistently found a MOMP isoform ($MOMP_{4.9}$) that, compared with the main isoform ($MOMP_{6.2}$), reacted with different sera or showed different intensity of recognition when the same sera was used. The different MOMP isoforms likely can be explained by posttranslational modifications without proteolytic processing. We speculate that in case of $MOMP_{4.9}$ posttranslational modifications, such as deamidation, result not only in the shift of the pI but also modulate its antigenic properties resulting in different immunoreactivity. Besides immunodominant proteins, our study revealed many Ags that were recognized by only some of the 38 sera. Although most of them turned out to be highly antigenic for individual sera, they possess only low overall reactivity. The analysis of the immunoreactive spots also revealed five human proteins that presumably reacted with cross-reactive or self-reactive serum Abs. Although in our study no association between Abs to self and disease sequelae was found, a role of these Abs in pathogenesis of any disease cannot be excluded.

After the identification of *C. pneumoniae* Ags, we compared the Ab-response pattern between groups of sera from subjects

with and without evidence for persisting *C. pneumoniae* infections. As a marker for persistent infections, we used the presence of *C. pneumoniae* DNA in PBMC of 23 healthy donors and in the coronary arteries of 11 heart transplant patients determined by *C. pneumoniae*-specific PCR. Among the 19 identified PCR-positive donors, we observed a different Ab-response pattern when compared with that of the 15 PCR-negative donors. Whereas RpoA, MOMP, YscC, Pmp10, PorB, Pmp21-m, GroEL, and Cpaf-c were strongly recognized by sera from PCR-positive donors, YscL, Rho, LcrE, and CpB0837 showed stronger reactivity with sera from PCR-negative donors. The physiological relevance of this finding is further underlined by the fact that the characteristic Ab-response pattern of PCR-positive donors is correlated with the altered protein expression known from for persistent *C. pneumoniae* infection in vitro. Using IFN-γ to mediate *C. pneumoniae* persistence, several studies have shown an up-regulation of RpoA, MOMP, PorB, and GroEL (43, 53). Likewise, for YscC an up-regulation is probable, because YscN, which is encoded by the same gene cluster, also showed increased protein expression (43). In our study, the highest increase in serum reactivity of PCR-positive donors was found toward Cpaf-c and RpoA. The latter protein was reported to be the most up-regulated protein during IFN-γ-mediated persistence (53). Consistent with the finding that the Ab response of PCR-positive donors reflects the protein expression of persisting *C. pneumoniae,* we found a lower reactivity toward proteins that were shown to be down-regulated during IFN-γ-mediated persistence, such as YscL and LcrE (53). In addition, down-regulation of CpB0837 is also most likely, because this protein is encoded by a gene cluster (54, 55) that showed a notably reduced transcription after addition of IFN-γ (56).

It has to be noted that a single PCR-based detection of *C. pneumoniae* DNA in PBMC can hardly distinguish persistent infection from active, recently acquired infection. However, in our study, the majority (9 of 14) of the PCR-positive healthy donors have been tested positive for *C. pneumoniae* DNA at two or more time points during 18 mo, which is rather unlikely due to two or even more recently acquired infections each coinciding with PBMC sampling. Consistent with this, none of the donors had a 4-fold rise of the *C. pneumoniae*-IgG titer as determined by MIF, arguing against acute infections. Furthermore, the detection of *C. pneumoniae* DNA in PBMC has been shown to coincide with the presence of these bacteria in atherosclerotic plaques (29, 57, 58) which is typically found in persistent infections. Compared with other studies that have used a single PCR analysis (reviewed in Ref. 59), we observed a high prevalence of *C. pneumoniae* DNA within the PBMC of healthy donors. This might be explained by the repetitive testing, which typically results in higher prevalence (20). Interestingly, among the PCR-positive healthy donors, the persistence-related Ab-response pattern was more pronounced in donors that repeatedly tested positive for *C. pneumoniae* DNA (data not shown).

Although the MIF is considered as the gold standard for *C. pneumoniae* serodiagnosis, the Ags detected on the surface of the *C. pneumoniae* EB by this assay are unknown. By comparing the reactivity of the Ags achieved within three groups of sera with different MIF titer, we identified 14 individual proteins that exhibited significantly higher reactivity with sera from MIF-positive donors (titer >64) compared with MIF-negative donors (titer <64). It is most likely that this group of proteins includes the Ags that contribute to MIF reactivity. This would fit with the finding that 9 of the 14 Ags identified here were demonstrated to be EB surface-exposed proteins, such as MOMP (both isoforms), OMP2, PorB, Pmp6, Pmp10, Pmp21, LcrE, and CpB0546 (60). Because the Ags recognized by the MIF were found to be characteristic for both the persistent and the nonpersistent infection status, it becomes clear that the MIF, even if properly performed, is not adequate at all to discriminate persistent from past infections.

Taken together, these data provide the first evidence for differences in the serological response toward *C. pneumoniae*, which were associated with the infection status of the donors. Whereas the Ab response of *C. pneumoniae* DNA-negative donors reflects the protein expression of *C. pneumoniae* covering the complete developmental cycle, which is likely the case in acute infections, *C. pneumoniae* DNA-positive donors showed specific reactivity toward Ags selectively up-regulated during *C. pneumoniae* persistence. Serological tests based on different selective combinations of specific rAgs, would to a major extent improve the serodiagnosis of *C. pneumoniae* infection, and might represent a tool to identify persistently infected patients.

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## Disclosures

The authors have no financial conflict of interest.

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