Molecular mimicry by *Mycoplasma pneumoniae* to evade the induction of adherence inhibiting antibodies

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Summary. Specific regions of adherence binding sites and epitopes of the P1 adhesin of *Mycoplasma pneumoniae* were synthesised as octapeptides and used as targets in a modified enzyme-linked immunosorbent assay. Acute phase and convalescent sera from 10 patients with *M. pneumoniae* infection were tested for antibody reactivity to these octapeptides. In convalescent sera, antibody activities were directed against octapeptides of the epitope regions, whereas no antibody activity was found in acute or convalescent sera to octapeptides of adherence-mediating binding sites. The non-responsiveness to adherence-mediating binding sites could be explained partially from the results of cross-reactivity experiments with adherence-inhibiting anti-P1 adhesin monoclonal antibodies (MAbs). Two of these MAbs showed cross-reactions with intracellular antigens of eukaryotic cell lines in immunofluorescence microscopy experiments. The cross-reacting antigens were isolated and characterised as glyceraldehyde-3-phosphate dehydrogenase and 2-phospho-D-glycerate dehydrogenase. Antigenic mimicry of eukaryotic structures by functional sites of the P1 adhesin of *M. pneumoniae* may influence the pathogenesis of *M. pneumoniae* infection.

Introduction

*Mycoplasma pneumoniae* is a common cause of community-acquired respiratory tract infection and c. 5–10% of *M. pneumoniae*-infected patients suffer from interstitial pneumonia. Adherence of *M. pneumoniae* to respiratory epithelial cells of the bronchial tract is a prerequisite for colonisation and propagation of the pathogen. The major P1 adhesin of *M. pneumoniae*, which is concentrated in a tip structure of mycoplasmal cells, mediates contact to epithelial cells. Establishment of anti-P1 protein monoclonal antibodies (MAbs) with adherence-inhibiting activity allowed the amino-acid sequences of the MAb binding sites to be characterised. At least three domains of the P1 protein—the N-terminal region and the D1- and D2-regions—are necessary to build up a tertiary adherence complex. However, on mapping the P1 primary amino-acid sequence for epitopes with patients' sera, several immunogenic regions were detected, none of which was identical to the binding sites of adherence-inhibiting MAbs.

This serological study was designed to clarify whether adherence-mediating sites are of low or no immunogenic capacity under natural conditions of human respiratory infection due to *M. pneumoniae*. Paired sera were collected from patients with respiratory symptoms who also had chest radiographs indicating an interstitial pneumonia and a positive culture of *M. pneumoniae* from nasopharyngeal aspirates. Acute phase and convalescent sera were tested for binding activities to oligopeptides which were synthesised according to known amino-acid sequences of epitopes or adherence-mediating binding sites of the P1 adhesin. Anti-P1 protein MAbs were also used to investigate possible cross-reactions between host antigens and *M. pneumoniae*.

Materials and methods

Clinical samples

*M. pneumoniae* was cultured from nasopharyngeal aspirates of patients with clinical signs of pneumonia and chest radiographs indicating an interstitial lung infiltration. The acute phase serum, together with the nasopharyngeal aspirate, was obtained from each patient on the day of admission to hospital and a second serum (convalescent sample) was collected 7–11 days later.
Serological tests

Paired sera were tested for anti-M. pneumoniae antibodies in two different enzyme-linked immunoassays (ELISA), based on sonicated M. pneumoniae whole cell antigen preparations or on the isolated P1 protein as antigen. Antibodies to defined octapeptides were also measured in these sera by the Pepsan ELISA (CRB, Cambridge, England). Octapeptides were synthesised on to polyethylene rods in duplicate according to amino-acid sequences of parts of the P1 protein which have been shown previously either to be involved in adherence (A1–A6) or to be epitopes (Nand B1–B4) (table). Patients’ sera or control sera (table) were pre-diluted 1 in 400 in blocking buffer—bovine serum albumin 1% w/v, ovalbumin 1% w/v and Tween 20 1% v/v in PBS (0.14 M NaCl, 0.01 M sodium phosphate, pH 7.2). Pre-diluted serum (100 µl) was incubated with each of the octapeptides for 1 h at 37°C. After incubation, the peptide trays were washed three times in Tween 20 0.05% in PBS and 150 µl of alkaline phosphatase-conjugated rabbit anti-human IgG (patients’ sera) or goat anti-mouse IgG (control MAbs), which had been diluted according to the manufacturers’ instructions (Dianova, Hamburg, Germany) were added. The trays were incubated for 1 h at 37°C and washed four times, before 150 µl of substrate solution (p-nitrophenyl phosphate 1 mg/ml in diethanolamine buffer, pH 9.6) were added, and the trays were incubated again for 1 h at 37°C. The absorbance of the coloured product was measured at 405 nm with an ELISA multiscan photometer (Titertek Multiscan MCC).

Cell lines and immunofluorescence microscopy

M. pneumoniae strain FH was grown at 37°C in Roux bottles in 50 ml of Hayflick’s modification of Edward’s medium. Glass-adherent mycoplasmas were harvested after 48 h by centrifugation, washed twice with PBS and stored at −20°C. Hybridoma cell lines (MAbs P1.26, P1.62, M51 and M58) were cultured in serum-free RPMI medium (RPMI-1640 containing 1 mM sodium pyruvate, penicillin 50 U/ml, streptomycin 50 µg/ml, 2 mM l-glutamine (Sigma) and BM-Condimed H1, Boehringer, 5%). The supernates were used without further concentration of MAbs in immunofluorescence tests and immunoblots.

The following immortalised cell lines were used: HeLa (human cervical carcinoma cell line, ATCC CCL 2); HEp-2 (epidermoid carcinoma, larynx, human cell line, ATCC CCL 23); HL-60 (promyelotic human leukemia cell line, ATCC CCL 240); MRC-5 (lung, diploid, human cell line, ATCC CCL 171); ACHN (renal adenocarcinoma, human cell line, ATCC CRL 1611); WiDr (colon, adenocarcinoma, human cell line, ATCC CCL218) and Vero (kidney, African green monkey cell line, ATCC CCL 81).

Cell lines were grown at 37°C in CO₂ 5% in air in Dulbecco’s modified Eagle’s minimal essential medium, supplemented with fetal calf serum 5%, gentamicin 50 µg/ml and glutamine 0.3 mg/ml. All eukaryotic cell lines were tested for mycoplasmal contamination by culture and the polymerase chain reaction (PCR) based on a common primer set which detects mycoplasmas in cultures. Contaminated cultures were cured by incubation with ciprofloxacin 50 µg/ml for three cell cycles. Cured cultures were re-tested for mycoplasmas by culture and PCR.

Mycoplasma-free cells were plated on glass cover slides, fixed for 10 min in acetone:ethanol (1:1) at −20°C and air dried. For indirect immunofluorescence staining, cells were first incubated with the different hybridoma supernates (MAbs P1.26, P1.62, M51 and M58) for 45 min at 37°C, washed with PBS and incubated for 30 min at 37°C with biotinylated goat anti-mouse antibody (Dianova.
Hamburg, Germany) diluted 1 in 100 in Tween 20 0.05% in PBS. The cells were washed, incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin, washed again and then evaluated with a Zeiss Axiophot microscope according to Fritsche et al. Control cells were incubated with RPMI medium alone in the first step.

Detection and characterisation of cross-reacting proteins

*M. pneumoniae* or eukaryotic cells were solubilised in electrophoresis sample buffer. One-dimensional analytical SDS-PAGE and immunoblotting of electrophoretically separated protein patterns were performed as described previously.

The supernate of sonicated and centrifuged (27 000 g for 1 h) eukaryotic HL-60 cells was used as a crude cytosolic protein fraction. For further characterisation of eukaryotic cell proteins, the cytosolic fraction was separated by SDS-PAGE, transferred from the gel to a PVDF (polyvinylidenedifluoride) membrane (Immobilon-P; Millipore), followed by immuno-staining with MAb supernates. Gas-phase sequencing of immobilised peptides was performed on Coomassie-stained bands which were cut out of transfer membranes. An N-terminal blocked protein was electro-eluted out of SDS-PAGE gels, cleaved chemically with cyanogen bromide, and the resulting protein fragments were separated electrophoretically and transferred to a PVDF membrane for sequencing of the N-termini of internal fragments.

Glyceraldehyde-3-phosphate dehydrogenase (GADH from rabbit muscle; EC 1.2.1.12) and 2-phospho-D-glycerate hydrolyase (enolase from rabbit muscle; EC 4.2.1.11) were purchased as reference proteins (Sigma).

Amino-acid sequences of the N-termini of the sequenced eukaryotic proteins were compared by means of the SwissProt protein sequence data library (DKFZ, Heidelberg).

Results

Antibody responses against epitopes and adherence-mediating sites of the P1 protein

Ten patients with a positive culture of *M. pneumoniae* and clinical and radiological signs of pneumonia were enrolled in the study. In acute phase sera, no or only low antibody activities were found in the P1 protein ELISA and in the *M. pneumoniae* whole-cell antigen ELISA, whereas all 10 convalescent sera showed high antibody responses in the P1 ELISA (OD\textsubscript{405} ≥ 1.4) and *M. pneumoniae*-ELISA (OD\textsubscript{405} ≥ 1.6). These paired sera were tested for antibody activity against amino-acid sequences that have been characterised as epitopes of the P1 protein (N and B1–4) or as adherence-mediating sites (A1–6). Acute phase sera showed no or only weak binding to the epitopes and no activity against the adherence-mediating epitopes. Compared to results with the control synthetic peptides K1 and K2 and those obtained with the acute sera, the convalescent sera showed elevated antibody activities to synthetic peptides of the epitope group. In contrast, antibody activity directed to the adherence-mediating group of synthetic peptides was not detectable in convalescent sera (fig. 1).

Cross-reactivity

The anti-P1 protein MAbs (P1.26, P1.62, M51 and M58) were tested for cross-reactions with different human tumour cell lines. MAbs P1.26 and P1.62 showed cross-reactions with all eukaryotic cell lines tested in immunofluorescence microscopy. Both MAbs showed homogeneous labelling of the cytoplasm excluding the nucleus area, as shown for MAb P1.62 with HeLa cells (fig. 2). In immunoblots MAb P1.62 showed cross-reaction with a 53-kDa protein and MAb P1.26 with a 36-kDa protein. In contrast to HeLa, MRC-5, ACHN, WiDr and HL-60 cell lines, the cross-reacting protein binding MAb P1.26 in Vero cells was slightly different with a mol. wt of c. 38 kDa (fig. 3). MAbs M51 and M58 showed no cross-reaction with eukaryotic cells in either immunofluorescence microscopy or immunoblots (results not shown).

Characterisation of cross-reacting proteins

The supernate of the crude cytoplasmic fraction of HL-60 cells was separated by SDS-PAGE and proteins were transferred to PVDF membranes (fig. 4). The 36- and 53-kDa proteins were cut out of the PVDF membrane for amino-acid gas-phase sequencing. The first 15 N-terminal amino acids of the 36-kDa protein were NH\textsubscript{2}-(1)-(G)-K-V-K-V-G-V-N-G-F-G-R-I-G-R whereas the N-terminus of the 53-kDa protein seemed to be blocked. Therefore, the 53-kDa protein was cut out of 10 SDS-PAGE gels and electro-eluted out of the gel matrix. Cyanogen bromide-cleaved fragments were separated and transferred to PVDF membranes to sequence the N-termini of the various 53-kDa fragments (fig. 5). The N-terminus of the 28-kDa fragment was not sequenceable. The 22-kDa fragment revealed the sequence NH\textsubscript{2}-R-I-G-A-E-V-Y-H-N-L-K-N-V-I-K.

Prediction and confirmation

The amino-acid sequences of the N-terminus of the 36-kDa protein and of the internal fragment of the 53-kDa protein were compared with known protein sequences in a protein sequence data library. The sequence of the 36-kDa protein revealed complete identity with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) and the internal sequence (N-terminal position 182) of the 53-kDa protein with 2-phospho-δ-glycerate hydrolyase (enolase; EC 4.2.1.11). To confirm the sequence homologies between the GAPDH and enolase with the
Fig. 1. Acute (lower insert) and convalescent (upper insert) sera of patients with *M. pneumoniae* pneumonia, tested for antibody (absorbance at 405 nm) to octapeptides of amino-acid sequences characterised as epitopes (N and B1-4) or as adherence-mediating sites (A1-6) of the P1 protein. The P1 protein diagram (uppermost) shows N-, A- and B-positions and membrane-associated helices M1-6. Octapeptides K1 and K2 were controls.

Fig. 2. Cross-reaction of the anti-P1 protein MAb P1.62 with HeLa cells in indirect immunofluorescence microscopy (left). As a control, HeLa cells were incubated without MAb suspension but with the indicator complex (right).

Amino-acid sequences of the 36- and 53-kDa proteins, respectively, defined enzymes were tested in immunoblots in parallel to human cell lines. The immunoblot in fig. 6 was developed with MAb P1.26. The MAb showed cross-reaction with GAPDH from rabbit muscles which was of the same mol. wt as the cross-reacting protein in HEp-2 cells. A slight difference in mol. wt was found on comparing the MAb P1.62 cross-reacting band of the human HEp-2 cell with the enolase isolated from rabbit muscle (fig. 7).

**Discussion**

The “gold standard” in the diagnosis of *M. pneumoniae* infection is still culture, although modern
Fig. 3. Cross-reactions in immunoblots of (A) MAb P1.62 and (B) MAb P1.26 with 53- and 36-kDa proteins of HL-60 cells, respectively. Amidoblack-stained protein patterns of 1, HeLa; 2, MRC-5; 3, ACHN; 4, WiDr; 5, Vero cell lines showed common protein bands within the 53- and 36-kDa mol. wt ranges.

Fig. 4. The positions of 1, 53-kDa; 2, 36-kDa proteins, were identified in immunoblots. The bands were cut out of a Coomassie blue-stained crude cytosol protein fraction of HL-60 cells (3) for N-terminal sequencing.

diagnostic techniques such as direct antigen tests and PCR allow more rapid diagnosis. To find out whether adherence epitopes are antigenic during the natural course of infection, patients with M. pneumoniae-positive nasopharyngeal aspirates were enrolled in this study. Mainly due to the problems encountered in cultivating the fastidious M. pneumoniae, only 10 cases could be enrolled in a 4-year study period. M. pneumoniae-positive patients showed increasing antibody activities in convalescent sera, compared to the acute sera, in ELISA tests with M. pneumoniae whole-cell antigen preparations or the isolated P1 protein as antigen. The delayed response demonstrated by elevated antibody activity in most convalescent sera but not in acute sera is in agreement with results from studies using the complement fixation test in M. pneumoniae serology. In parallel with the elevated antibody activity to whole antigens found in convalescent sera, elevated antibody responses to octapeptides of the P1 protein epitope group were also detected in the Pepscan ELISA. In contrast, no antibodies were detected against adherence-mediating sites of the P1 adhesin in either acute or convalescent sera.

To find out whether the adherence-mediating regions might share antigenic determinants with host cells, different eukaryotic cell lines were tested for cross-reaction with four MAbs recognising different adherence-mediating binding sites. Two of the four MAbs showed distinct cross-reactions with the cytoplasmic enzymes GAPDH and enolase, which are both enzymes of the glycolytic pathway. Recently, it was shown that GAPDH is distributed homogeneously throughout the cytoplasm, excluding the nucleus and vesicles, and is associated with the actin cytoskeleton. Antigenic similarities between M. pneumoniae molecules and human GAPDH and enolase might be responsible for non-responsiveness or for a self-limitation of the immune response during natural infection with M. pneumoniae. In the pathogenesis of M. pneumoniae infection, the failure of induction of adherence-inhibiting antibodies might explain the prolonged isolation of M. pneumoniae from the res-
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Fig. 5. SDS-PAGE patterns of HL-60 cell proteins (1), the cyanogen bromide-cleaved 53-kDa protein (2), and high-mol. wt (MW) and low-mol. wt (LMW) markers in a 10% gel.

Fig. 6. Binding of MAb P1.26 to the P1 protein of M. pneumoniae (1) and cross-reactions with the 36-kDa protein of HeLa (2) and HL-60 (3) cells and GAPDH isolated from rabbit muscle (4) in immunoblots (W). A: amidoblock-stained protein profiles.

The emerging questions are: (i) is it possible to break the non-responsiveness; (ii) would such adherence-inhibiting antibodies protect against subsequent infections; and (iii) what would be the side-effects of these "auto-immune" antibodies? The development of auto-antibodies during M. pneumoniae infection is a well known phenomenon. Lind et al. have shown that more than half of patients suffering M. pneumoniae infection with positive complement fixation titres (≥ 512) were also positive for auto-antibodies to the mitotic spindle apparatus of eukaryotic cells and for cold agglutinin. In contrast to the recognised clinical association between cold agglutinin autoantibodies and intravascular haemolysis, anti-lymphocyte antibodies and antibodies to brain, lung, cardiolipin, smooth muscle and the mitotic spindle apparatus in patients with M. pneumoniae infections have not been shown to have an obvious role in pathogenicity. There may be a difference in
respect of autoantibody induction to ζ-enolase. Approximately 40% of sera with anti-neutrophil cytoplasmic autoantibodies (ANCA) from patients with clinically proven vasculitis show binding to enolase. Moreover, a role for mycoplasma structures as auto-antigens in chronic vascular diseases, e.g., rheumatoid arthritis, and in perinatal disorders in man and in great apes has been discussed. In the light of these findings, consideration of the possibility of a vaccine that could break non-responsiveness and protect the host from *M. pneumoniae* infection would have to take into account the potential side-effects of auto-antibody induction. Under natural conditions, *M. pneumoniae* has adapted to its host in a perfect way. During first and subsequent infections, the adherence process to respiratory epithelial cells cannot be blocked because of the hosts’ inability to establish adherence-inhibiting antibodies.

### References


