Topological mapping of the P1-adhesin of *Mycoplasma pneumoniae* with adherence-inhibiting monoclonal antibodies

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Five adherence-inhibiting monoclonal antibodies (mAbs) were used for topological mapping of the binding sites of the 169 kDa membrane-integrated adhesin of *Mycoplasma pneumoniae*. Antibody binding sites were characterized using overlapping synthetic octapeptides. Three regions of the protein seem to be involved in adherence: the N-terminal region [N-reg, epitopes beginning at amino acid (aa) 1 to aa 14 and aa 231 to aa 238, respectively]; a domain (D1) approximately in the middle of the molecule (beginning at aa 851 to aa 858 and aa 921 to aa 928); and a domain (D2) closer to the C-terminus (beginning at aa 1303 to aa 1310, aa 1391 to aa 1398 and aa 1407 to aa 1414). Each of the mAbs P1.26 and P1.62 reacted with two primary amino acid sequences. Both antibodies bound to the D1 region, but mAb P1.62 showed additional binding to a sequence (aa 231 to aa 238) near the N-terminus, and mAb P1.26 reacted with a second epitope in the D2 domain (aa 1303 to aa 1310). Such dual binding by the two antibodies suggests that in the native protein the epitopes are composed of two sequences which are located on two different sites of the molecule (D1/N-reg and D1/D2, respectively). In a competitive ELISA test using native *M. pneumoniae* cells as antigen, both the mAb directed to the N-terminus (aa 1 to aa 14) and mAbs directed to those epitopes nearest to the C-terminus (aa 1391 to aa 1398 and aa 1407 to aa 1414) were able to prevent binding of mAbs P1.26 and P1.62 to the native cells. The results of epitope mapping and inhibition tests suggest a close spatial relationship of the domains N-reg, D1 and D2 in forming a functionally active adherence binding site. Furthermore, the outermost epitopes of the domains involved seem to be located on top of folded loops of the protein molecule.

Introduction

The human respiratory pathogen *Mycoplasma pneumoniae* adheres strongly to erythrocytes (Feldner *et al.*, 1979; Baseman *et al.*, 1982), hamster tracheal cells (Hu *et al.*, 1976; Gabridge *et al.*, 1978) and human lung fibroblasts (Gabridge *et al.*, 1979). The major adhesin mediating contact to these cells (P1-protein) is concentrated at the tip-like structure of the organism (Hu *et al.*, 1982; Feldner *et al.*, 1982). Monoclonal antibodies (mAbs) binding to the P1-protein inhibit the attachment of the pathogen to red blood cells and hamster tracheal cells (Hu *et al.*, 1982; Morrison-Plummer *et al.*, 1986). The mature P1-adhesin consists of 1567 amino acids (Jacobs *et al.*, 1987; Dallo *et al.*, 1988; Inamine *et al.*, 1988). One of the primary sequences of the adherence-mediating regions has been located near to the C-terminus by screening a recombinant DNA expression library of *M.*

pneumoniae with an anti-P1 adherence-inhibiting mAb (Dallo *et al.*, 1988). Using peptide patterns of cyanogen-bromide-cleaved P1-protein we recently found two additional and different binding sites of adherence-inhibiting mAbs. One is located near the N-terminus and the other approximately in the middle of the amino acid sequence (Jacobs *et al.*, 1989). From these results, we speculated that the native attachment structure might be composed of several surface-exposed loops. To obtain further information on the topographical arrangement we used two adherence-inhibiting mAbs which bound to two epitopes, each on separate domains. The results of inhibition studies with these and other adherence-inhibiting mAbs are reported here and their implications are discussed.

Methods

Peptide synthesis. Solid-phase peptide synthesis based on the use of N^ε-(9-fluorenylmethoxycarbonyl) (Fmoc) amino acids (Dryland &

Abbreviations: aa, amino acid; mAb, monoclonal antibody; TFA, trifluoroacetic acid.

Sheppard, 1986; Sheppard, 1986) was used for the preparation of synthetic antigens. The peptides were synthesized in two different ways: (i) for the production of adequate amounts of the synthetic N-terminus, a semi-automatic peptide synthesizer (Biolyne 4175; Pharmacia/LKB) was used; (ii) the epitope scanning kit (CRB) producing overlapping synthetic octapeptides was used according to the method of Geysen *et al.* (1984) for analysis of the mAb binding sites within the preselected P1-protein regions N-reg (aa 172 to aa 343), D1 (aa 841 to aa 941) (Jacobs *et al.*, 1989) and D2 (aa 1241 to aa 1441) (Dallo *et al.*, 1988).

The column-synthesized N-terminus of the mature P1-protein was cleaved from the resin (Ultrosyn A; Pharmacia/LKB) by treatment with 95% (v/v) trifluoroacetic acid. The released peptide was precipitated and washed repeatedly with diethyl ether. The raw peptide preparation was dried, and purified by HPLC using a reverse-phase column (TSK ODS-120T, C₁₈, 5 µm, 4.6 × 250 mm; LKB). A linear gradient (0.6% B min⁻¹) was applied [eluant A, 0.1% TFA; eluant B, acetonitrile/water (80:20, v/v) 0.08% TFA] flow rate 1 ml min⁻¹. The eluate was monitored at 215 nm. The homogeneous peptide peak fractions were pooled and dried using a 'Speed-vac' centrifuge. The pin-immobilized overlapping peptides were acetylated with acetic anhydride, the side-chain protecting groups were cleaved and, after extensive washing, the peptides, still bound to the solid phase, were dried according to the manufacturer's (Cambridge Research Biochemicals) recommendations.

Preparation of adherence-inhibiting mAbs. Two different antigens were used for immunization: (i) viable glass-adherent *M. pneumoniae* cells (strain FH) cultured in Hayflick's modification of Edward's medium (Hayflick, 1965); (ii) isolated and purified P1-adhesin (Jacobs *et al.*, 1988). Two 3-month-old male Balb/c mice were injected intraperitoneally with freshly harvested mycoplasma cells (1 mg total protein per injection) or isolated P1-protein (Jacobs *et al.*, 1988; 100 µg per injection). The antigens were used without any adjuvant. The immunizations (intraperitoneal) were repeated four times at weekly intervals and 3 to 4 d before spleen cells were removed at *post mortem* for fusion. The somatic-cell fusion with X63-Ag8.653 myeloma cells was done according to the method of De St Groth & Scheidegger (1980). Several hundred viable hybridoma clones were recovered from both fusions. The secreted antibodies were screened for: (i) recognition of the P1-protein and sonicated mycoplasma cells in ELISA systems (Jacobs *et al.*, 1986); (ii) binding to the denatured and electroblotted P1-protein using Western immunoblotting (Towbin *et al.*, 1979); (iii) inhibition of adherence of sheep red blood cells to mycoplasmas (Jacobs *et al.*, 1985); and (iv) binding to the tip-structure of the organism in an immunofluorescence test using glass-adherent native mycoplasmas (Feldner *et al.* 1982). The appropriate hybridomas were cloned by using a limiting-dilution technique. Two clones of the anti-P1 fusion [P1.26 and P1.62(F2); Jacobs *et al.*, 1989] and two clones of the anti-mycoplasma fusion (M51 and M58) fulfilled the requirements mentioned above and were cultured *in vivo* in mice for ascites production.

For the preparation of site-specific mAbs the synthetic N-terminus of the mature P1-protein was cleaved from the synthesizer resin and purified by HPLC. The oligopeptide was used in free form for the immunization of Balb/c mice (Atassi, 1986). The mice were immunized with 100 µg purified peptide, injected intraperitoneally together with an aluminium hydroxide adjuvant (Alu-Gel-S; Serva). Four injections were given at monthly intervals. Somatic-cell fusion was done as described above. Twenty-two antibody-producing hybridomas survived the selection. The antibodies were screened for binding to the homologous peptide and were tested for activity in the various assays described above.

Epitope mapping with overlapping peptides. Ascites fluids from the different hybridoma clones were diluted in blocking buffer [1% BSA,

1% ovalbumin, 1% Tween 20 in PBS (0.14 M-NaCl, 0.01 M-sodium phosphate, pH 7.2)], and incubated overnight with the pre-blocked solid-phase (pin)-immobilized octapeptides at 4 °C. The peptide-pins were washed with PBS/Tween 20 and incubated with an alkaline-phosphatase-conjugated secondary antibody (goat anti-mouse immunoglobulins; Dianova, FRG) diluted in blocking buffer. After the final washing, the pins were incubated in substrate solution (10 mg ml⁻¹ *p*-nitrophenyl phosphate in diethanolamine buffer, pH 9.6) for 1 h at 37 °C. The A₄₀₅ was determined with a Titertek Multiscan.

Competition ELISA. *M. pneumoniae* cells were cultured in Roux flasks with Hayflick's modification of Edward's medium (Hayflick, 1965) for 48 h at 37 °C. Glass-adherent cells were scraped off and washed with PBS. The cell suspension was adjusted to a protein concentration of 100 µg ml⁻¹ determined according to the method of Peterson (1977). Microtitre plates were pretreated with poly(L-lysine) (50 µg per well) and glutaraldehyde (100 µl of a 20%, v/v, solution in deionized water) (McEachran & Irvin, 1986). Samples (100 µl) of the cell suspension were incubated on these microtitre plates for 2 h at 37 °C under gentle rotation. The plates were washed with pre-warmed PBS and remaining aldehyde groups were blocked with 3% BSA/PBS for 1 h at 37 °C. Unlabelled mAb (100 µl per well) was added and incubated for 2 h at 37 °C under maximum antigen saturation conditions (the relative mAb concentration that is necessary to block all accessible binding sites). Biotin-labelled mAb (500 ng in 20 µl per well) was then added without washing and further incubated for 1 h at 37 °C (Jackson *et al.*, 1982; Stähli *et al.*, 1983). The plates were washed three times with PBS/0.05% Tween 20. The binding of the biotin-labelled mAb was detected with streptavidin/peroxidase (Sigma; 200 ng per well). Substrate (H₂O₂/tetramethylbenzidine) was added and the colour development was measured as the A₄₅₀ after 30 min incubation at room temperature.

Results

Screening for adherence-inhibiting mAbs

Two out of 300 hybridomas from fusion experiments with viable *M. pneumoniae* cells (M51 and M58) and two out of 800 hybridomas from the P1-protein immunization (P1.26 and P1.62) showed adherence-inhibiting activity. These mAbs also recognized isolated P1-protein and sonicated mycoplasma cells as antigen in ELISA tests. Additionally, they showed binding to the P1-protein in Western immunoblots. In the immunofluorescence test with native organisms, these mAbs gave distinct fluorescence located at the tip-like structure of the mycoplasmas, indicating P1-specificity on intact cells. Anti-peptide mAb aN was specific for the homologous peptide and also showed fluorescence on the tip-structure of *M. pneumoniae*, indicating a cell-surface location of the N-terminus (aa 1 to aa 14) of the mature membrane-integrated P1-protein. mAb aN strongly inhibited the adherence of sheep red blood cells to *M. pneumoniae*.

Epitope mapping with overlapping synthetic peptides

The adherence-inhibiting mAbs were tested for binding to overlapping octapeptides mimicking three regions of

Fig. 1. ELISA activity of the mAb P1.26 (ascites) with synthetic, overlapping solid-phase octapeptides of the amino acid sequence of domain D1 (aa 841 to aa 1011). The insert shows the binding sites for the different mAbs within the primary amino acid sequence of the mature P1-protein. For mAb aN the location of the synthetic antigen used for immunization is shown. The epitopes of the mAbs resulted from peptide-ELISA experiments. The solid areas indicate predicted membrane-associated helices according to Eisenberg *et al.* (1984).

the mature P1-protein: (i) the N-terminal region (Thr-173 to Tyr-344); (ii) domain D1 (Asn-841 to Arg-1011); and (iii) domain D2 (Asn-1241 to Pro-1441).

An example of an antigenic profile resulting from the peptide-ELISA is shown for mAb P1.26 (IgG₁κ) with domain D1 (Fig. 1). The antibody activity is clearly limited to one peptide in D1 [NH₂-(921)-N-A-L-S-F-T-N-K]. The same antibody also binds to a peptide sequence in domain D2 [NH₂-(1303)-D-V-V-G-V-G-R-L]. The mAb P1.62 (IgG₁κ) binds to the sequence NH₂-(851)-E-N-H-T-K-F-T-S in D1 and to the octapeptide NH₂-(231)-E-V-K-K-K-S-D-S in the N-terminal region. mAbs M58 (IgG₁κ) and M51 (IgG_{2b}κ) showed binding to D2 on NH₂-(1391)-S-V-N-P-K-M-V-R and NH₂-(1407)-N-E-Q-S-L-G-L-R respectively.

Anti-peptide mAb aN (IgG₁κ), which is directed against the synthetic N-terminus of the mature P1-protein, also showed strong adherence-inhibiting activity but possessed no antigenic site in D1 and D2 when tested with these short linear peptides.

Competition-ELISA

To investigate the epitopes for the different mAbs with regard to the native conformation of the P1-adhesin, a competitive ELISA was done. The results are shown in Table 1. The dilution of the pre-incubated mAb (ascites fluid) was pre-tested for maximal competition with the

Table 1. Competitive ELISA for epitope mapping of the P1-protein adherence structure using viable *M. pneumoniae* cells and adherence-inhibiting mAbs

Inhibition (I) was defined arbitrarily as follows: I ≤ 20%, no inhibition (0); I 21 to 50%, partial inhibition (+/-); I > 50%, inhibition (+). Undisturbed binding of each mAb was used as a non-competition control, and is defined as having 100% binding efficiency and producing 0% inhibition. The test included self-competition controls.

Pre-incubation with mAb	Inhibition of probed biotin-labelled mAb				
	aN	P1.62	P1.26	M51	M58
aN (N-term)	+	+	+	+/-	+
P1.62 (N-reg/D1)	0	+	+	0	0
P1.26 (D1/D2)	0	+/-	+	0	0
M51 (D2)	0	+	+	+	0
M58 (D2)	+/-	+	+	+/-	+

homologous mAb in the ELISA (mAb dilutions ranged from 1:10 to 1:200). The assay also included non-competitive controls: each biotinylated mAb was analysed for binding without pre-incubation of unlabelled antibodies. The preincubated mAbs aN (aa 1 to aa 14) and M58 (anti-D2, aa 1391 to aa 1398) inhibited binding of all other mAbs totally or partially, but were inhibited only by each other. mAb M51 (anti-D2, aa 1407 to aa 1413) interfered with binding of mAb P1.26 (anti-D1, aa 921 to aa 928; anti-D2, aa 1303 to aa 1310) and mAb

P1.62 (anti-N-terminal region, aa 231 to aa 238; anti-D1, aa 851 to aa 858), but was itself inhibited only by mAbs aN and M58. The mAbs P1.26 and P1.62 were inhibited by all other antibodies. In pre-incubation, these two mAbs inhibited only each other.

Discussion

The results suggest that at least three regions of the P1-protein are necessary to build the tertiary structure that mediates adherence of *M. pneumoniae* to host cells. Recently, Dallo *et al.* (1988) reported an amino acid sequence, NH₂-(1324)-G-I-V-R-T-P-L-A-E-L-L-D-G, which showed binding activity with an adherence-inhibiting mAb. This sequence is positioned between the epitopes of our adherence-inhibiting mAbs P1.26 [NH₂-(1303)-D-V-V-G-V-G-R-L] and M58 [NH₂-(1391)-S-V-N-P-K-U-V-R] of the D2-binding site. At least two other quite different regions of this high-molecular-mass protein are involved in the adherence mechanism. Using cyanogen bromide fragments of the P1-protein and mAbs, we recently described antigenic regions near the N-terminus (beginning with the amino acid phenylalanine-177) and another region located approximately within the middle of the P1-protein (D1 region: beginning with the amino acid tryptophan-642) (Jacobs *et al.*, 1989). Testing overlapping peptides in an ELISA, the binding sites of the two mAbs P1.62 and P1.26 have now been identified precisely. Interestingly, both antibodies each reacted with two epitopes: mAb P1.26 bound to D1 and D2 and mAb P1.62 to N-reg and D1.

All three domains (N-reg, D1 and D2 region) are defined by the following computer-predicted membrane-associated helices (see Fig. 1): (i) N-reg to D1 by three hydrophobic sequences, aa 421 to aa 447, aa 471 to aa 491 and aa 733 to aa 753; (ii) D1 and D2 by the predicted helix aa 1047 to aa 1066.

Berzofsky & Schechter (1981) and Atassi (1984) showed that antigenic sites of proteins are mainly composed of separated primary sequences brought together by protein-folding in the native state, thus forming a discontinuous functionally active site. This process might also be responsible for the dual binding sites of the mAbs P1.26 and P1.62.

The results of the epitope mapping and of the inhibition studies elucidate some aspects of the topology of the (functionally active) adherence binding site. The close association of the domains D1 with D2, on one hand, and of D1 with N-reg, on the other, is suggested by the dual binding of the mAbs P1.26 and P1.62, respectively. Additional information is given by the inhibition tests with native P1-protein of living *M. pneumoniae* cells. Antibodies against two regions [N-term

Fig. 2. Possible topographical arrangement of the adherence-mediating binding sites of the P1-protein of *M. pneumoniae*. The binding sites of the adherence-inhibiting mAbs (Fig. 1) and the results of the competitive ELISA (Table 1) show a close relationship between D1 (located near the middle of the protein) and D2 (located near the C-terminus) and of the N-terminal region with both D1 and D2 as a result of the competition patterns of the mAbs P1.26 (a), P1.62 (b), aN (c), M51 (d) and M58 (e).

(aa 1 to aa 14) and D2 (aa 1391 to aa 1398)] were able to prevent the binding of mAbs to the other five epitopes. This effect can be interpreted in different ways. (i) mAb M58 and mAb aN binding sites could be located at the top of surface-exposed protein loops, whereas binding sites of mAb P1.26 and P1.62 are positioned in a depression of the surface of the folded protein. Therefore, it is possible that mAb M58 and mAb aN could block the entrance of the other three mAbs. (ii) Binding of antibody mAb aN and mAb M58 could also provoke conformational changes within the tertiary structure (Guirakhoo *et al.*, 1989), so that the two binding sites of mAbs P1.26 and P1.62 are no longer surface exposed. (iii) Stone & Nowinski (1980) discussed a third possibility: after monovalent binding of IgG antibodies to a molecule, a considerable amount of molecular rotational freedom is retained, which might also disturb the binding of mAbs P1.26 and P1.62.

The reciprocal inhibition of the mAbs P1.62 (N-reg/D1) and P1.26 (D1/D2) suggests a close association of these sites. The inhibition could be caused by steric competition for the limited space available within the suggested surface depression of the protein, rather than by the closeness of the different epitopes. Pre-incubation with mAb aN also blocked mAb M51 and mAb M58 binding. Pre-incubation with mAb M58 was able partially to block the mAb aN, whereas mAb M51 did not affect binding of mAb aN. These findings suggest not only that the D1 domain is located near the N-terminal region and the D2 domain, but also that the N-terminal region must be in close contact with the D2 region. In this hypothetical arrangement, at least three

different loops are brought together by protein folding in the native P1-protein (Fig. 2).

Apparently, the adherence mechanism of *M. pneumoniae* to target cells is more complicated than first experiments suggested (Inamine *et al.*, 1988). The data support the idea of a three-dimensional arrangement of different P1-membrane protein segments necessary to form an effective adherence structure. Whether this arrangement exists permanently on the native protein or is only formed after contact with receptor structures is as yet unknown. The requirement for energy for adherence to surfaces (Feldner *et al.*, 1981), and the lack of binding at 4 °C (Feldner *et al.*, 1979) could be taken as support for the latter possibility.

Furthermore, it is not known whether the interaction between binding site and receptors is dependent more on variable factors, such as charge patterns and hydrophobic interactions, or on defined amino acid sequences of the P1-protein with carbohydrate receptors on the host cells. The variety of receptors described in the literature (Gabridge *et al.*, 1978, 1979; Geary & Gabridge, 1987; Loomes *et al.*, 1985) suggests to some extent an involvement of the flexible mechanisms mentioned above. In this way, the binding sites of the extracellular parasite *M. pneumoniae* could adapt themselves to the various structures of the host cells of the respiratory tract.

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