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Interaction between the P1 protein of *Mycoplasma pneumoniae* and receptors on HEp-2 cells

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The human pathogen *Mycoplasma pneumoniae* can cause atypical pneumonia through adherence to epithelial cells in the respiratory tract. The major immunogenic protein, P1, participates in the attachment of the bacteria to the host cells. To investigate the adhesion properties of P1, a recombinant protein (rP1-II) covering amino acids 1107–1518 of the P1 protein was produced. This protein inhibited the adhesion of *M. pneumoniae* to human HEp-2 cells, as visualized in a competitive-binding assay using immunofluorescence microscopy. Previous studies have shown that mAbs that recognize two epitopes in this region of P1 also reduce *M. pneumoniae* adhesion. Therefore, peptides covering these epitopes, of 8 and 13 aa, respectively, were synthesized to further investigate the adhesion region. None of these synthetic peptides reduced the binding of *M. pneumoniae* to the receptors on the host cells. Instead, 10 overlapping synthetic peptides covering the whole of rP1-II were evaluated in the competitive-binding assay using immunofluorescence microscopy. A reduction in the number of *M. pneumoniae* microcolonies was seen, which was confirmed for five peptides using a POLARstar OPTIMA reader to measure fluorescence intensity. The number of *M. pneumoniae* microcolonies adhering to the host cells was significantly reduced by these five peptides. Further investigations showed that inhibiting peptide 7 (amino acids 1347–1396) of the major adhesin protein P1 bound directly to host receptors, suggesting that the observed *M. pneumoniae*-inhibiting peptides occupied HEp-2 receptors, which are otherwise available for P1-mediated *M. pneumoniae* adhesion.

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INTRODUCTION

Mycoplasma pneumoniae is a human pathogen that colonizes the upper and lower respiratory tract, causing pneumonia. It attaches to ciliated epithelial cells in the respiratory tract, where it induces ciliostasis that protects the mycoplasma from removal by the mucociliary clearance mechanism of the host (Waites & Talkington, 2004). A key characteristic of *M. pneumoniae* is the lack of a cell wall, which allows exchange of different components between the host membrane and the mycoplasma membrane after adhesion (Razin *et al.*, 1981). Apart from respiratory-tract cells, *M. pneumoniae* adheres to many different types of eukaryotic cells *in vitro*, e.g. HEp-2, HeLa, macrophages and erythrocytes (Razin *et al.*, 1981).

Abbreviations: DAPI, 6-diamino-2-phenylindole; IMF, indirect immunofluorescence microscopy; TBTU, *o*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate.

M. pneumoniae is elongated and consists of a longer tail-like rear end, a thicker body part and a frontal attachment organelle. This organelle is composed of a network of proteins, of which the adherence accessory proteins are thought to confer the tip structure, and to underlie mycoplasma mobility as well as the clustering of a network of adhesins at the tip (Bredt, 1979; Waites & Talkington, 2004). The P1 protein, which is mainly concentrated at the tip, is one of the major adhesins in *M. pneumoniae*, and the loss of P1 through mutation results in reduced adherence to host cells (Krause, 1996). Furthermore, a fragment of the C-terminal part of the P1 protein has been shown previously to be immunogenic, as sera from *M. pneumoniae* patients show a reaction with this fragment (Drasbek *et al.*, 2004). Recently, it has been seen that anti-P1 antibodies reduce the gliding speed of *M. pneumoniae*, thus hampering the mobility of the bacterium and possibly its ability to find suitable host adhesion receptors (Seto *et al.*, 2005).

Attachment of bacteria to host cells is one of the key steps of infection and often relies on an interaction between bacterial adhesins and oligosaccharides on the host cells. These interactions are usually mediated by binding of lectins present on the bacterial surface to oligosaccharide chains bound to glycoproteins and glycolipids on eukaryotic cells (Thomas & Brooks, 2004). This is probably also the case for *M. pneumoniae* attachment, since reduced adhesion of *M. pneumoniae* has been demonstrated when human alveolar epithelial cells (A549) are pre-treated with tunicamycin, which degrades oligosaccharides (Thomas & Brooks, 2004). In addition, other studies have shown that the adhesion of *M. pneumoniae* to tracheal epithelial cells decreases by 50–65 % when they are treated with neuraminidase, which cleaves terminal acylneuraminic residues from oligosaccharides, glycoproteins and glycolipids. Likewise, heat, methiolate, glutaraldehyde and formalin also inhibit the adhesion of *M. pneumoniae* to eukaryotic cells (Gabridge & Taylor-Robinson, 1979; Razin *et al.*, 1981).

Adhering mycoplasmas contain several adhesins, and *M. pneumoniae* expresses at least three different adhesins, termed P1, P30 and P116, suggesting that there are multiple receptors for *M. pneumoniae* on the host cells (Razin & Jacobs, 1992; Svenstrup *et al.*, 2002). Monospecific antibodies raised against P116 block the adhesion of *M. pneumoniae* to HEp-2 cells, as do antibodies against the P30 protein (Baseman *et al.*, 1987). It is, however, unclear whether this inhibition is direct or results from steric hindrance of neighbouring structures. Recently, elongation factor Tu and the pyruvate dehydrogenase E1 β subunit have been shown to bind fibronectin, which is usually found in the extracellular matrix of eukaryotic cells (Dallo *et al.*, 2002). In addition, the surface protein P65 contains an Arg-Gly-Asp (RGD) domain, raising the possibility that *M. pneumoniae* attaches to binding sites for fibronectin on the host cell (Krause, 1996).

Recently, it has been shown that antibodies against the C-terminal part of the *M. pneumoniae* P1 protein and the homologous *Mycoplasma genitalium* MgPa protein inhibit host-cell adhesion of *M. pneumoniae* and *M. genitalium*, respectively (Svenstrup *et al.*, 2002). mAbs that recognize epitopes in this region (Dallo *et al.*, 1988; Jacobs *et al.*, 1990) also inhibit the binding of *M. pneumoniae* to host cells. However, no inhibition is observed using antibodies that target the N-terminal parts of the proteins. In mycoplasma-free extracts, P1 binds to host cells, suggesting a direct role for P1 in host receptor binding (Krause, 1998; Razin & Jacobs, 1992). Thus, the anti-P1 antibody inhibition of adhesion could originate from blocking of the adhesion region of P1. However, simple steric hindrance by the antibodies could also explain the observed inhibition. Therefore, in the present study, we applied a competitive-binding assay to determine the attachment-mediating region of the C-terminal part of P1. A recombinant protein covering this part of P1 (rP1-II; Fig. 1), as well as several synthetic overlapping peptides covering the same region, was utilized, and both rP1-II and some of the peptides inhibited *M. pneumoniae* adherence to receptors on HEp-2 cells. Direct binding of one peptide to the host cells was detected using biotinylated peptides, suggesting a direct interaction between the P1 protein and a host cell receptor. Furthermore, this receptor was shared between the peptide and rP1-II, as the binding of the biotinylated peptide was inhibited in a concentration-dependent manner by unmarked peptides and the rP1-II protein.

METHODS

Preparation of HEp-2 cells for infection with *M. pneumoniae*. The human epithelial cell line HEp-2 (ATCC) was cultured in TPP tissue-culture flasks containing growth medium [RPMI 1640 medium (Gibco-BRL) with 25 mM HEPES-buffer (0.01 M HEPES, 0.15 M NaCl, pH 7.2) and 10 % sterile filtered fetal calf serum (FCS)], with 10 μg gentamicin ml^{-1}. One day prior to *M. pneumoniae* infection,

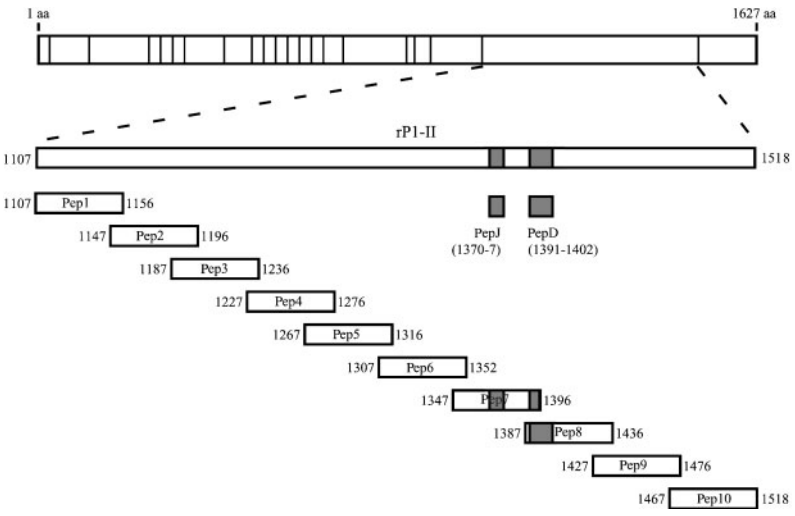

Fig. 1. Schematic illustration of the P1 amino acid sequence. The positions of the UGA codons (tryptophan) are indicated by black lines. The recombinant rP1-II protein covers part of the C-terminal region of the protein (amino acids 1107–1518), while the 12 synthetic peptides cover the same region. The grey boxes denote the two inhibiting antibody-binding regions, pepJ and pepD (Dallo *et al.*, 1988; Jacobs *et al.*, 1990).

HEp-2 cells were trypsinated (0.25 % trypsin in PBS with 0.02 % EDTA) and plated in growth medium with 0.05 % penicillin (100 U ml^{-1}). Each well of either a 16-well Lab-Tek chamber slide (Nunc) or a black 96-well Nunclon cell-culture plate (both with optical bottoms) contained 3750 cells per well, while 18 750 cells per well were plated in a 24-well plate containing glass coverslips. The cells were incubated overnight in 5 % CO_2 at 37 °C before *M. pneumoniae* infection.

Preparation of *M. pneumoniae* and *M. genitalium* for infection of HEp-2 cells. *M. pneumoniae* strain FH and *M. genitalium* G37 (ATCC) were cultured in 10 ml SP4 medium (Tully *et al.*, 1979) in TPP tissue-culture flasks and incubated at 37 °C. After growth for 48 h, the medium changed colour from red to orange, indicating the exponential growth phase (Clausen *et al.*, 2001). The mycoplasmas were scraped into 10 ml growth medium containing penicillin (100 U ml^{-1}). To reduce the number of self-aggregating features of *M. pneumoniae*, the suspension was sheared through a 27 gauge needle five times and adjusted to a concentration of approximately 9×10^7 colour changing units ml^{-1} before infection of the HEp-2 cells.

Generation of the recombinant protein rP1-II. The recombinant protein *M. pneumoniae* rP1-II aa 1107–1518 and the recombinant *M. genitalium* protein rMgPa aa 628–1350 were produced as described previously (Svenstrup *et al.*, 2002). To reduce the concentration of urea in the purification buffer, the proteins were first diluted to 0.2 mg ml^{-1} in growth medium and then dialysed against the same medium with 10 μg gentamicin ml^{-1} for 2 h at 4 °C.

Synthesis of peptides. Two epitopes, pepJ (Jacobs *et al.*, 1990) and pepD (Dallo *et al.*, 1988), of 8 and 13 aa, respectively, were synthesized (Fig. 1). Additional peptides of 44–52 aa covering rP1-II were designed with an overlap of 6–10 aa (Fig. 1), together with a random peptide with the sequence RIYKGVIQAIQKSDEGHPFR-AYLESEVAISEELVQKYSNSALGHVNCTIKELRRLFLVDDLVDSLK. Peptides were synthesized stepwise on a fully automated peptide synthesizer ABI 433 (Applied Biosystems) using the Fmoc strategy. Peptides were synthesized on a TentaGel S RAM resin [Fluka; s (substitution) 0.2 mM g^{-1}] to generate peptide amides. Fmoc-protected amino acids in suitable side-chain-protected forms as well as the coupling reagent *o*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) were obtained from Fluka. After completion of assembly, peptides were cleaved from the resin, with simultaneous side-chain deprotection, using trifluoroacetic acid and triisopropylsilane/water as scavenger. Filtrates were concentrated *in vacuo* and peptides precipitated with ether. Precipitates were lyophilized from acetic acid/water. Molecular mass verification was made by MS using MALDI-TOF for all peptides except peptide 1, the size of which was instead verified by SDS-PAGE, since it fragmented during MS.

Biotinylation of peptides. Biotinylation was carried out after completion of peptide assembly while the peptide, with protected side groups, was still attached to the resin, using biotin (Sigma-Aldrich) and TBTU as coupling reagents. The biotinylated peptide was cleaved from the resin and treated as described above.

Competitive-binding assay with rP1-II. The dialysed rP1-II recombinant protein (10, 20, 40 or 80 μl) was incubated with HEp-2 cells grown in chamber slides for 30 min at 37 °C. The *M. pneumoniae* RPMI suspension (50 μl per well) was added and incubated overnight at 37 °C. The HEp-2 cells were washed twice in PBS and fixed in 100 % methanol at 4 °C for 1 min. To detect the adhering mycoplasmas, the infected HEp-2 cells were incubated with primary antibodies (PabFH or PabG37) diluted 1 : 500 for 30 min at 37 °C (Svenstrup *et al.*, 2002), washed with PBS and incubated with 100 μl per well of secondary FITC-conjugated 'Affinipure' goat anti-rabbit (GaR) IgG (H + L) (Jackson Immuno Research Laboratories) diluted 1 : 100 in PBS with 0.002 % Evans blue, for 30 min at 37 °C. The wells were washed twice with PBS before attached *M. pneumoniae* were visualized using a fluorescence microscope.

Competitive-binding assay with synthetic peptides. The *M. pneumoniae* suspension was incubated with each of the synthetic peptides (20 μg ml^{-1}; ~10^{15} molecules) for 30 min at 37 °C. HEp-2 cells were incubated with the mycoplasma peptide suspension (50 μl per well) overnight. The samples were prepared for indirect immunofluorescence microscopy (IMF) as described in the competitive-binding assay with rP1-II.

IMF. The samples prepared for the adhesion detection and the competitive-binding assays were investigated by IMF. A drop of antifade solution [*p*-phenyldiamine dihydrochloride (1 μg ml^{-1}) in 10 % PBS and 90 %, v/v, glycerol, pH 9.0] was placed between the slide and the coverslip. Fluorescence microscopy was performed with a Leitz DMR fluorescence microscope (Leica).

Indirect immunofluorescence for fluorometric measurement by POLARstar. The *M. pneumoniae* suspension was mixed with each of the synthetic peptides (20 μg ml^{-1}) or a dilution series was made with *M. pneumoniae* to obtain a standard curve. HEp-2 cells were plated in black 96-well Nunclon cell-culture plates with optical bottoms, as described above, and incubated either with the mycoplasma peptide suspension or with diluted mycoplasmas (50 μl per well) overnight. The samples were prepared as described above for the rP1-II competitive-binding assay for IMF, with the exception that PBS with 6-diamino-2-phenylindole (DAPI; 1/1000) was added instead of antifade to each well before the fluorescence measurement was carried out using a POLARstar OPTIMA reader (BMC Labtech). Emission and excitation filters were 488 and 520 nm for FITC and 526 and 532 nm for DAPI, respectively.

Immunofluorescence with fluorescent beads. HEp-2 cells were prepared as described above and incubated with biotinylated peptide 1 or peptide 7 (80 μg ml^{-1}) for 30 min at 37 °C. In the peptide competition experiment, HEp-2 cells were preincubated with unmarked peptides or proteins for 30 min before the 30 min incubation with biotinylated peptides. The cells were fixed as described for the competition assay with rP1-II. To detect receptor-bound peptides, the cells were incubated with 10 μl NeutrAvidin-coated yellow/green fluospheres (Invitrogen), diluted 1 : 10 in PBS for 10 min at 4 °C, washed and investigated using IMF.

Statistical analysis. In each experimental round, all samples were tested in duplicate or triplicate, as well as being repeated on different experimental days. Results are expressed as mean values with SDs. *P* values were calculated using Student's *t* test for normally distributed data (fluorescent intensity measurements), while the non-parametric Mann–Whitney test was used for the remaining data (microcolony counting) with a significance level of $P<0.05$ (two-tailed).

RESULTS

Competitive-binding assay with rP1-II

The immunogenic P1 protein is clustered at the tip organelle and participates in pathogen–host interactions (Baseman *et al.*, 1982). The recombinant rP1-II protein contained a 6 × His tag, which was used for nickel-affinity purification under denaturing conditions in a urea-containing buffer

(Svenstrup *et al.*, 2002). However, urea is toxic to HEp-2 cells, so rP1-II was diluted to 0.2 mg ml^{-1} and subjected to dialysis against growth medium for 2 h prior to use. Four different concentrations of rP1-II were incubated with HEp-2 cells for 30 min at 37 °C before live *M. pneumoniae* were added. The infected cells were incubated overnight at 37 °C, washed in PBS and fixed with methanol. Adherent *M. pneumoniae* were detected with pabFH, a polyclonal antibody raised against *M. pneumoniae* whole-cell proteins, and visualized using FITC-conjugated IgG antibody. To fluorescently visualize HEp-2 cells, the fixed cells were counterstained with Evans blue (red fluorescence, Fig. 2a) prior to immunofluorescent detection of attached *M. pneumoniae*. To estimate the inhibition of adhesion, the bound *M. pneumoniae* microcolonies were counted on individual HEp-2 cells that were not in contact with other cells. Each microcolony was counted as one, regardless of the individual size of the microcolonies. In the positive control without recombinant proteins, an average of 25 microcolonies per HEp-2 cell (range 16–33 microcolonies per HEp-2 cell) was counted (Fig. 2b). Preincubation with rP1-II prior to addition of *M. pneumoniae* to the HEp-2 cells clearly reduced the number of *M. pneumoniae* microcolonies associated with the HEp-2 cells (Fig. 2c) in a dose-dependent manner, as 10, 20, 40 and 80 μl rP1-II (2 μg ml^{-1}) resulted in 17.3, 16, 8.5 and 6 *M. pneumoniae* microcolonies per HEp-2 cell, respectively. Furthermore, shortening the *M. pneumoniae* incubation time to 1 h showed a similar reduction in the number of microcolonies, suggesting that host cell receptors are persistently occupied by the recombinant proteins (data not shown). In contrast, no inhibition of *M. pneumoniae* adhesion was observed using a recombinant protein (rMgPa) from the closely related *M. genitalium* (data not shown). Thus, rP1-II seems to compete with *M. pneumoniae* for binding to receptors on HEp-2 cells.

Competitive-binding assay with two synthetic peptides covering the two known epitopes

To further analyse the region of attachment, two synthetic peptides (pepJ and pepD; grey boxes in Fig. 1) which were recognized by inhibiting antibodies were tested in the competitive-binding assay. The number of microcolonies was counted and compared to the positive control without peptides. Both peptides showed a slight reduction in the number of *M. pneumoniae* microcolonies, 21.8 % for pepJ and 16.9 % for pepD, compared to the positive control without peptides. However, neither peptide reduced the binding of *M. pneumoniae* to HEp-2 cells significantly (P=0.27 and 0.41, respectively), as was also the case for a synthetic peptide spanning a region from pepJ to pepD (data not shown). To ascertain that the counting of microcolonies on the individual cells was not biased by the researcher, the fluorescence intensity of bound *M. pneumoniae* was measured using the POLARstar OPTIMA in black 96-well plates. The nuclear fluorescent stain DAPI was included as a measure of the number of host cells. The fluorescence measurements were fairly stable between wells and plates, showing a uniform distribution of HEp-2 cells in the plates. The amount of bound mycoplasma was calculated using a standard curve made from a dilution series of *M. pneumoniae*. Only slight variations in calculated *M. pneumoniae* concentration were seen when pepJ and pepD were tested for inhibition, confirming the manually counted results (data not shown).

Competitive-binding assay with 10 synthetic peptides

Because no significant competition was seen between *M. pneumoniae* and the two short peptides (pepJ and pepD), another approach to narrow down the adhesion region was utilized. Ten peptides (44–52 aa) covering the same region of P1 as rP1-II were designed with 6–10 aa overlaps (Fig. 1). These peptides were then tested in the competitive-binding assay to estimate the inhibitory effect of each peptide on *M. pneumoniae* adherence. As a negative control, the closely related *M. genitalium* was included. After incubation with the peptides, adherent *M. pneumoniae* on HEp-2 cells were detected as described for rP1-II, while *M. genitalium* were detected using an antibody raised against *M. genitalium* whole-cell proteins.

The attachment of mycoplasmas to host cells was estimated by counting microcolonies on solitary HEp-2 cells and by measuring fluorescence intensity using the POLARstar as before. Between 49 and 94 HEp-2 cells were selected for each peptide in order to estimate the number of *M. pneumoniae* microcolonies per HEp-2 cell (Fig. 3). Likewise,

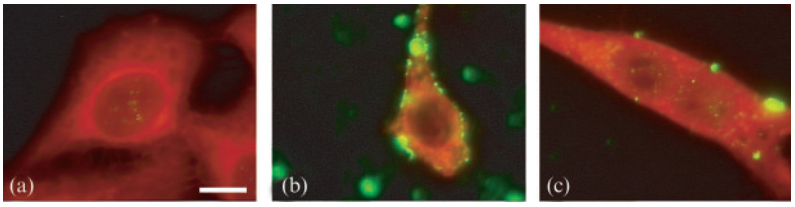

Fig. 2. Competitive-binding assay, with the recombinant rP1-II protein visualized using immunofluorescence microscopy. Evans blue stains the entire HEp-2 cell red. The *M. pneumoniae* microcolonies are visualized with the primary antibody pab(FH) and a FITC-conjugated (green fluorescent) secondary anti-rabbit IgG antibody. (a) Uninfected HEp-2 cells; (b) HEp-2 cells incubated with *M. pneumoniae*; (c) HEp-2 cells incubated with 80 μl rP1-II before infection with *M. pneumoniae*. Bar, 10 μm.

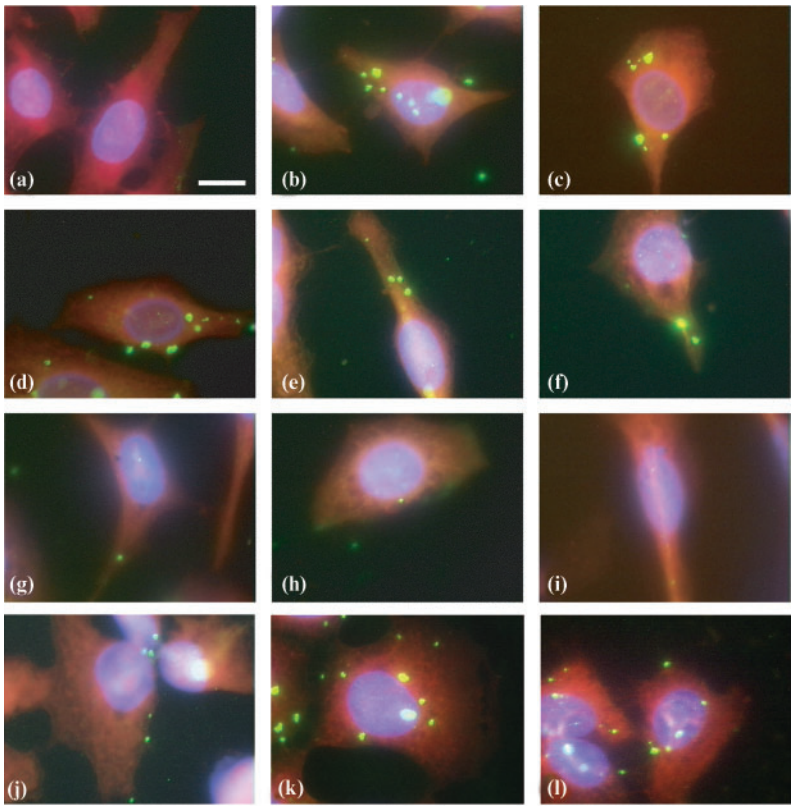

Fig. 3. Competitive-binding assay with 10 synthetic overlapping peptides using immunofluorescence microscopy. The HEp-2 cells are stained with Evans blue (red) and DAPI (blue). The *M. pneumoniae* microcolonies attached to the HEp-2 cells are detected by the polyclonal antibody pab(FH) and FITC-conjugated secondary antibody (green dots). (a) Negative control showing uninfected HEp-2 cells with no binding of pab(FH); (b) positive control without peptides showing *M. pneumoniae* microcolonies; (c–l) preincubation with peptides 1–10, respectively. Bar, 10 μm.

M. genitalium microcolonies on 17–26 HEp-2 cells were counted for each peptide. In the negative control (Fig. 3a), no *M. pneumoniae* microcolonies were detected, while a few mycoplasma microcolonies were detected with peptides 3–8 (Fig. 3e–j) compared to the positive control (Fig. 3b). In contrast, the numbers of microcolonies after competition with peptides 1–2 and 9–10 (Fig. 3c–d and k–l) were closer to the number in the positive control. These results were obtained after preincubating the peptides with *M. pneumoniae* before HEp-2 cell infection. However, preincubating the peptides with HEp-2 cells and/or reducing the *M. pneumoniae* incubation time to 1 h did not alter the results noticeably.

All peptides significantly reduced the adhesion of *M. pneumoniae,* but to varying degrees. A histogram of the mean number of microcolonies showed a bell-shaped curve (Fig. 4, Table 1) with peptides 1–3 and 9–10 exhibiting the smallest reduction in adherence compared to peptides 4–8, with peptide 7 (amino acids 1347–1396) showing the greatest inhibition, 78.9 % ($P<0.0001$). The adherence of *M. genitalium* to HEp-2 cells was not inhibited by any of the 10 peptides (data not shown). A slightly higher number of *M. genitalium* microcolonies was detected when the peptides were added.

The fluorescence intensity of bound *M. pneumoniae* in competition with the 10 synthetic peptides was also measured using the POLARstar OPTIMA to obtain an objective measure of bound *M. pneumoniae*. The amount of bound mycoplasma was calculated using a standard curve, as described above. Eight peptides (peptides 2–9) could compete with *M. pneumoniae* in binding to the HEp-2 cells with a reduction of 2–16.6 % (Table 2). Five of

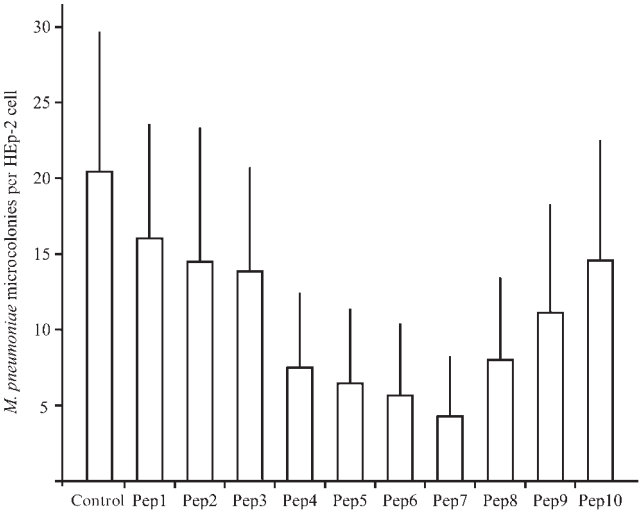

Fig. 4. Summary of the competitive-binding assay with *M. pneumoniae* and the 10 synthetic peptides. The histogram shows the mean ± SD (bars) of the number of microcolonies after incubation with the peptides.

Table 1. Inhibition of *M. pneumoniae* adhesion by the synthetic P1-derived peptides

The mean number of *M. pneumoniae* microcolonies per HEp-2 cell was counted after IMF. *n*, Number of individual HEp-2 cells counted.

Peptide	Mean count of *M. pneumoniae* microcolonies	Relative inhibition (%)	*n*	*P* value (two-tailed)
Control	20.4±9.2		71	
Peptide 1	16.0±7.5	21.6	81	0.001
Peptide 2	14.5±8.8	28.9	69	0.0001
Peptide 3	13.9±6.8	31.9	68	0.0001
Peptide 4	7.5±4.9	63.0	74	0.0001
Peptide 5	6.4±4.9	68.6	94	0.0001
Peptide 6	5.7±4.7	72.0	91	0.0001
Peptide 7	4.3±3.9	78.9	56	0.0001
Peptide 8	8.0±5.4	60.8	48	0.0001
Peptide 9	11.1±7.1	45.6	67	0.0001
Peptide 10	14.5±7.9	28.9	56	0.0001

these (peptides 4–7 and peptide 9) reduced binding significantly ($P<0.05$, Student's *t* test), and this corresponded to the results of microcolony counting. No inhibition of *M. genitalium* was observed (data not shown).

Binding of peptides to HEp-2 cells

As the peptides clearly reduced the attachment of *M. pneumoniae* to host cells, it was interesting to see whether the peptides associated directly with host cells, thereby causing a reduction in *M. pneumoniae* adherence. To investigate the binding of the peptides to HEp-2 cells, two peptides were selected, peptide 1, which caused the smallest inhibition, and peptide 7, which caused significant *M. pneumoniae* inhibition. These peptides were biotinylated at the N-terminal end and incubated with HEp-2 cells for 30 min at 37 °C. The cultures were washed and methanol-fixed, and bound peptides were visualized using NeutrAvidin-coated yellow/green fluospheres (beads) (Fig. 5). No fluorescence was seen from the biotinylated peptides without beads (Fig. 5a), while the beads showed a sporadic binding pattern without biotinylated peptide (Fig. 5b). The clustering of the beads on the HEp-2 cells after preincubation with peptide 1 suggested an interaction between the peptide and the HEp-2 cells (Fig. 5c). A more striking interaction was observed with peptide 7 (Fig. 5d), which correlates with the ability of the peptide to inhibit *M. pneumoniae* adhesion.

The number of beads per HEp-2 cell was counted on 18–31 HEp-2 cells for peptides 1 and 7 and the control. For the control, 4.6±4.1 beads per HEp-2 cell without biotinylated peptide were found. In comparison, the mean numbers of beads bound with peptide 1 and peptide 7 were 15.8±8.4 and 59.3±21.2 beads per HEp-2 cell, respectively. This suggests that peptide 7 interacts with HEp-2 cells through a specific receptor. To investigate whether the binding of peptide 7 was genuine and shared the same host receptor as P1 and thereby also *M. pneumoniae*, a peptide-competition

Table 2. FITC fluorescence intensity of *M. pneumoniae* on host cells

The fluorescence intensity of FITC-marked *M. pneumoniae* microcolonies was normalized to the positive control. *n*, Number of wells scanned.

Peptide	Normalized fluorescence	*n*	*P* value (two-tailed)
Control	1.00±0.13	18	
Peptide 1	1.07±0.24	15	0.357
Peptide 2	0.98±0.13	15	0.666
Peptide 3	0.97±0.21	15	0.823
Peptide 4	0.83±0.14	19	0.001*
Peptide 5	0.85±0.16	16	0.005*
Peptide 6	0.85±0.15	18	0.004*
Peptide 7	0.88±0.08	18	0.002*
Peptide 8	0.98±0.15	18	0.595
Peptide 9	0.90±0.14	16	0.046†
Peptide 10	1.01±0.12	15	0.750

*$P<0.01$.
†$P<0.05$.

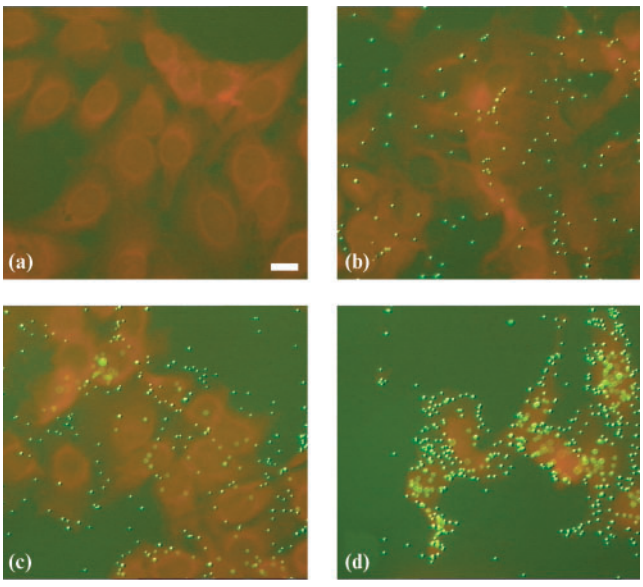

Fig. 5. Binding of peptides to HEp-2 cells. The biotinylated peptides attached to the HEp-2 cells were detected with NeutrAvidin-coated yellow/green fluospheres. (a) Negative control with peptide 7 and without yellow/green fluospheres shows no fluorescence; (b) HEp-2 cells without peptides show a diffuse distribution of the yellow/green fluospheres; (c) peptide 1 associates with the HEp-2 cells as seen by the binding of the beads; (d) peptide 7 strongly clusters the beads on the HEp-2 cells. Bar, 10 μm.

assay was performed. Close-to-confluent HEp-2 cells were pre-incubated with varying concentrations of unmarked peptide 7 for 30 min, and then challenged with biotinylated peptide 7 for 30 min. As almost no solitary HEp-2 cells were present in these preparations, the total number of bound beads was counted in consecutive pictures covering the entire well in a vertical line, thus generating higher numbers than reported for the initial experiment. The average number of beads per microscope frame decreased as the concentration of unmarked peptide 7 was increased (Table 3). Likewise, increasing amounts of rP1-II decreased the number of biotinylated peptide 7 on the host cells. Neither a non-specific control peptide nor rMgPa from *M. genitalium* inhibited the binding of biotinylated peptide 7 (data not shown). These results show a pronounced direct interaction between the synthetic peptide 7 and the HEp-2 cells, an interaction that could be blocked by rP1-II, suggesting that the peptide inhibits *M. pneumoniae* adhesion by occupying receptors otherwise recognized by the P1 protein.

DISCUSSION

The P1 protein is one of the major *M. pneumoniae* adhesins, expressed all over the surface of the pathogen but clustered at the tip structure. This clustering is thought to play an important role in cytadherence of *M. pneumoniae* to host cells, since *M. pneumoniae* lacking the P1 protein on the membrane fail to adhere to the host cells (Layh-Schmitt & Harkenthal, 1999). In addition, P1 is the most immunogenic *M. pneumoniae* protein, and antibodies recognizing this protein are always found in human serum samples from *M. pneumoniae*-infected patients (Hu *et al.*, 1983; Jacobs *et al.*, 1985, 1989). The C-terminal part of the protein has been found to be the immunogenic region, as determined using human sera, and monospecific and monoclonal antibodies (Chaudhry *et al.*, 2005; Drasbek *et al.*, 2004). Recently, using monospecific antibodies, it has been found that the C-terminal part of P1 is surface-exposed (Svenstrup *et al.*, 2002). Furthermore, in the present study, a direct interaction between the P1 protein and host cell receptors was found, since the recombinant protein rP1-II clearly reduced adhesion of *M. pneumoniae*.

Table 3. Competitive binding of synthetic peptides and recombinant proteins

The mean number of beads associated with a confluent layer of host cells per microscopic frame is given. The total number of bound beads was counted in consecutive pictures covering the entire well in a vertical line to ensure randomness. The control was counted after incubation with 80 μg ml^{-1} biotinylated peptide 7. Unmarked rP1-II and peptide 7 inhibited biotinylated peptide 7 attachment. *n*, Number of frames counted.

Peptide	Concentration (μg ml^{-1})	Mean number of beads per frame	*n*
Control		205.2 ± 89.9	22
rP1-II	100	95.6 ± 37.4	11
	50	114.7 ± 42.2	8
	25	131.0 ± 51.6	8
	10	136.5 ± 32.5	8
Peptide 7	100	134.3 ± 83.0	10
	50	158.3 ± 51.5	8
	25	172.0 ± 49.2	5
	10	191.3 ± 72.7	14

The model of P1 contains several surface-exposed loops, which have been suggested to form three domains, thereby building a tertiary adherence complex (Jacobs *et al.*, 1989, 1990, 1995). Epitope mapping of the P1 protein has led to the suggestion that the N-terminal region and two additional domains, D1 and D2, are extracellular and arranged in close proximity (Fig. 6). This would enable the three different loops to form a triangular structure, which might exhibit a stronger adherence capacity due to co-operative binding to the receptor molecule (Gerstenecker & Jacobs, 1990; Razin & Jacobs, 1992). However, the adhesion-mediating protein sequences might not be identical to the sequences described as immunogenic epitopes. Indeed, adhesion-inhibiting mAbs targeting sequences not recognized by human serum samples have been described (Jacobs *et al.*, 1990). In the present study, the region of the P1 protein which mediates adhesion to the host cells was mapped to the D2 domain. Sequences in this region (Fig. 6) have previously been linked to cytadherence, since mAbs targeting these sequences reduce *M. pneumoniae* adhesion to the host cells (Dallo *et al.*, 1988; Jacobs *et al.*, 1990). This inhibition is probably caused by steric hindrance of the large antibodies, because no significant reduction in the adhesion of *M. pneumoniae* to the HEp-2 cells was seen with pepJ and pepD, which cover these epitopes. In addition, peptide 7, which showed the greatest inhibitory effect, included pepJ and part of pepD, suggesting that the host receptor binding sequence of the P1 protein is to be found in the C-terminal half of rP1-II.

The adhesion region of *M. pneumoniae* was narrowed using 44–50 aa synthetic peptides (Fig. 6). Five peptides inhibited the adhesion of *M. pneumoniae* to HEp-2 cells significantly, as measured by both manual counting and automatic measurements. However, the amount of reduction determined by the two methods differed. Manual counting of *M. pneumoniae* only included microcolonies associated with solitary host cells, giving a precise measure of attached *M. pneumoniae* microcolonies. However, these counts could be biased by the researcher, and therefore the unbiased fluorometric POLARstar measurements of the entire well of a black 96-well plate were included. Some basal background fluorescence was anticipated as *M. pneumoniae* attaches directly to plastic. These microcolonies would be unaffected by the host-receptor-blocking peptides, leading to lower relative reductions in *M. pneumoniae*-generated fluorescence. The synthetic peptides

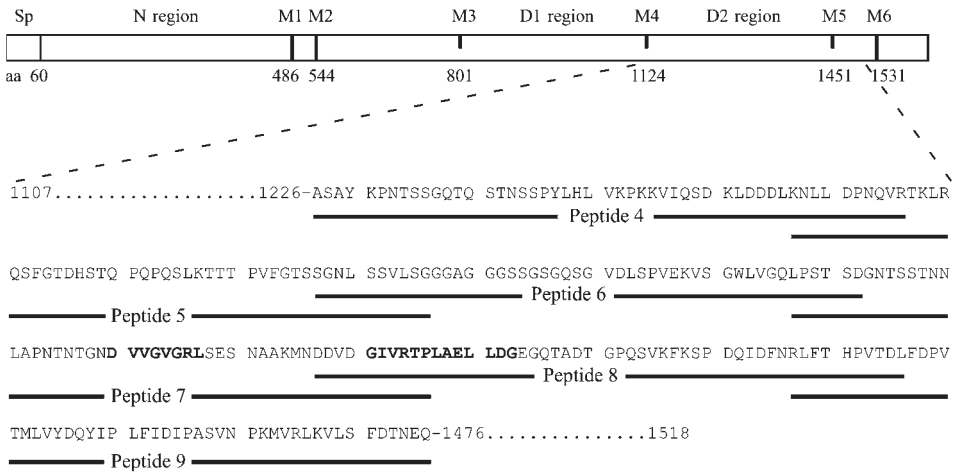

Fig. 6. Membrane-associated domains and the rP1-II region. Adapted from Jacobs *et al.* (1990) and Eisenberg *et al.* (1984). The membrane-associated domains were identified using the program http://www.expasy.org/tools/pscale/Hphob.Eisenberg.html using the following parameters: window size, 9; relative weight for window edges, 100 %. The black lines M1–M6 indicate the membrane-associated domains, and the aa number marks the first amino acid in the transmembrane-associated domain. The rP1-II region (amino acids 1107–1518) is shown, and the sequences of peptides 4–9 are included. The sequences of pepJ (Jacobs *et al.*, 1990) and pepD (Dallo *et al.*, 1988) are in bold type. Sp, signal peptide.

inhibited the adhesion of *M. pneumoniae* to host cells to varying degrees. A similar graduation of adhesion inhibition has been observed elsewhere in the investigation of the outer-membrane protein P5-homologous fimbrin adhesin protein from *Haemophilus influenzae*, in which both short and long peptides covering the protein show differing degrees of inhibition of bacterial adhesion (Novotny *et al.*, 2000). The inhibition region, peptides 4–9, is flanked by the two membrane-associated regions, M4 and M5, in the extracellular D2 region (Fig. 6), suggesting that the D2 region binds directly to the host cell. Indeed, peptide 7 of P1 was found to bind a specific host receptor that was shared with the P1 protein, suggesting that the inhibiting peptides function through the blockage of specific host cell receptors. Similarly, a fragment of the P29 adhesin from *Mycoplasma fermentans* inhibits the adhesion of the bacteria to HeLa cells through the blockage of host cell receptors (Leigh & Wise, 2002).

In the present study, only a partial inhibition was seen, although an excess of peptides was added. This might be due to the difference in size and mobility of *M. pneumoniae,* which is larger and more mobile than the small and immobile peptides. Additionally, the observed partial inhibition could reflect the interaction of other *M. pneumoniae* proteins with receptors on the host cell, as a stronger inhibition of adhesion was seen previously when antibodies raised against P116 and the C-terminal part of P1 were combined (Svenstrup *et al.*, 2002). This P116 protein has been identified elsewhere as a host-receptor-binding protein under the name of P2 together with the P1 protein and HMW3 (Krause *et al.*, 1982), and it has been reported that the extracellular protein fibronectin is bound by elongation factor Tu and the pyruvate dehydrogenase E1 β subunit from *M. pneumoniae* (Dallo *et al.*, 2002). It is therefore likely that adhesion of *M. pneumoniae* to host cells is a more complex process than the receptor-mediated binding of peptide 7 of P1 to host cells, as described in the present study. To obtain a more complete understanding of the host–*M. pneumoniae* interaction, a model system of human ciliated respiratory cells should be used.

Such an understanding could be used to block host cell receptors to which pathogenic microorganisms adhere. This may be considered as an alternative strategy to prevent colonization by the pathogen. Indeed, a synthetic peptide mimicking an adhesion epitope of the oral bacterium *Streptococcus mutans* inhibits the colonization of tooth surfaces by this micro-organism (Younson & Kelly, 2004). Thus, the introduction of *M. pneumoniae*-derived peptides that block human adhesion receptors might in the future reduce or even prevent *M. pneumoniae* infections.

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