

MICROBIAL PATHOGENICITY

Adhesion and inhibition assay of *Mycoplasma genitalium* and *M. pneumoniae* by immunofluorescence microscopy

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Adhesion of *Mycoplasma pneumoniae* and the closely related *M. genitalium* to HEp-2 cells was investigated. The main surface proteins known to be involved in adhesion are P1 of *M. pneumoniae* and its homologue, MgPa, of *M. genitalium*. Both proteins are also immunodominant proteins. Protein P116 is another immunodominant protein of *M. pneumoniae*. These immunogenic proteins were investigated for their surface exposure and involvement in adhesion to host epithelial cells. Immunofluorescence microscopy (IFM) was used to detect *M. pneumoniae* and *M. genitalium* adhering to HEp-2 cells. Monospecific antibodies were produced against fragments of the surface proteins lacking tryptophan stop codons and were used for adhesion detection, surface exposure and adhesion inhibition IFM assays. Three monospecific antibodies were made against MgPa covering regions in the N-terminal, the middle and the C-terminal part; two monospecific antibodies were produced against P1 covering regions of the N- and the C-terminal part and one monospecific antibody was made against most of P116. Only the C-terminal parts of P1 and MgPa were surface exposed and blocking of these regions with the monospecific antibody resulted in inhibition of cytoadsorption. Protein P116 was shown to be surface exposed and an essential protein involved in adhesion because the anti-P116 antibody prevented attachment of *M. pneumoniae* to the HEp-2 cells independently of P1. This study adds to the understanding of the molecular biology of *M. genitalium* and *M. pneumoniae* and presents a method to study the proteins involved in adhesion of these mycoplasmas.

Introduction

Mycoplasmas are eubacteria lacking a cell wall. They are the smallest known self-replicating organisms with a cell diameter of only 300 nm [1] and a genome size in the range of 580 kb for *Mycoplasma genitalium* [2] to 1350 kb for *M. mycoides* [3]. The mycoplasma genome is AT-rich [4] and uses the UGA stop codon to encode tryptophan [5].

M. pneumoniae and the closely related *M. genitalium* are both human pathogens with a specialised tip structure. They use the tip for adhesion to ciliated epithelial host cells, gliding motility [6] and cell division [7, 8]. *M. pneumoniae* is known to colonise ciliated lung epithelia cells and cause an atypical

pneumonia. The less well-characterised *M. genitalium* is believed to be involved in non-gonococcal urethritis [9] and pelvic inflammatory disease [10].

Triton X-100 treatment of whole cells of *M. pneumoniae* revealed a filamentous, cytoskeleton-like network of proteins (the Triton shell) in the differentiated tip [11, 12] analogous to eukaryotic cytoskeleton proteins [13]. The Triton shell proteins are composed primarily of high mol. wt proteins (HMW) 1–3. These proteins are thought to connect with surface proteins and accessory proteins during adhesion as an energy-demanding process [14]. The HMW proteins probably have a scaffolding function that includes the localisation and anchoring of the adhesins to the tip as well as the maintenance of the attachment organelle [15].

the immunodominant protein but both adhesins elicit immunological responses in man [16–18]. P1 and P30 are clustered specifically in the tip structure of the attachment organelle [19, 20] and the adhesins are directly involved in binding to host epithelial receptors [21].

A very immunogenic protein of 116 kDa (P116) has also been characterised [22]. Whether this protein is involved in cytodhesion is not known, but the protein is demonstrated to be a surface protein by trypsin degradation [22].

Nearest-neighbour analyses with cross-linking reagents have shown the organisation of the proteins of *M. pneumoniae* associated with the membrane. The analyses revealed that P1 was located in a complex with adhesin P30, the 40- and 90-kDa surface proteins (ORF-6 cleavage products) and the HMW proteins 1 and 3 in the cell membrane. However, surface protein P116 was not detected as a part of the P1-multiprotein complex [23, 24].

Ultrastructural studies have indicated that *M. genitalium* is flask-shaped and possesses a cytoskeleton-like structure as seen in *M. pneumoniae* [25]. Only few proteins involved in cytodhesion have been characterised in *M. genitalium*. The surface antigen, MgPa (150 kDa), is the major adhesin of *M. genitalium* and it is homologous to the major P1 adhesin of *M. pneumoniae* (51.7% amino acid (aa) identity) [26]. Furthermore, MgPa is the most immunogenic protein of *M. genitalium* [27–29]. Recently, two putative cytodherence-related proteins of *M. genitalium*, P32 and P69, have been described but the functional activities of these proteins are still unproven [30]. The hypothetical protein, MG075, of *M. genitalium* is homologous to P116 of *M. pneumoniae* (52.2% aa identity) [2] but expression of MG075 has not been demonstrated [31]. A second immunodominant protein, P114, was identified as being encoded by MG192 (114-kDa protein of the MgPa-operon) and not related to P116 [29, 32].

Recombinant proteins covering specific domains of MgPa of *M. genitalium* as well as of P1 and P116 of *M. pneumoniae* were produced to study attachment-mediating regions. Polyclonal monospecific antibodies were produced by immunising rabbits with the recombinant proteins. Indirect immunofluorescence microscopy (IFM) was used to investigate whether these monospecific antibodies were able to inhibit the cytodesorption of *M. genitalium* and *M. pneumoniae*.

Materials and methods

Micro-organisms

M. genitalium G37 and *M. pneumoniae* FH (ATCC, MD, USA) were cultured in 100 ml of SP-4 medium

[33] in TTP tissue-culture flasks (Medi Cult, Copenhagen, Denmark) and incubated at 37°C. After growth for 48 h, the medium changed colour from red to orange which indicated an exponential growth phase and the cells were harvested. Mycoplasma cells attached to the bottom of the culture flask were scraped off in phosphate-buffered saline (0.01 M sodium phosphate, NaCl 0.85%, pH 7.4; PBS) and the cells were pelleted by centrifugation at 10 000 *g* for 30 min. The cells were resuspended in 4 ml of PBS and centrifuged in eppendorf tubes at 20 000 *g* for 15 min. The supernate was removed and the pellets were stored at –70°C [29].

Extraction of mycoplasma DNA

One pellet of *M. genitalium* and *M. pneumoniae* from cultivation in 20 ml of SP-4 medium [33] (5 pellets/100 ml) was individually dissolved in 160 ml of Tris-EDTA buffer [34] with 40 µl of proteinase K (Boehringer Mannheim GmbH, Germany) 200 µg/ml. The solution was incubated at 37°C for 30 min after incubation at 55°C for 30 min and then boiled for 5 min. The extracted DNA was stored at –20°C.

Generation of recombinant proteins

Expand™ High Fidelity PCR System (Boehringer Mannheim) was used to amplify the genes encoding the *M. genitalium* protein, MgPa, and the *M. pneumoniae* proteins, P1 and P116. Primers to amplify the *mgpa*, *p1* and *p116* genes were designed from the nucleotide sequences with GenBank accession nos M31431 (strain G37), M18639 (strain M129) and Z71425 (strain M129). Nested PCR was done with the *mgpa* and *p1* gene because of homologous repetitive sequences scattered in the genomes of *M. genitalium* and *M. pneumoniae*, respectively (the nested primers are shown in Table 1). The positions of the primers for the production of recombinant proteins were limited by tryptophan stop codons. Three fragments of *mgpa* were amplified covering nucleotides (nt) 229–960 (no. I), nt 2488–3027 (no. II) and nt 3223–4332 (no. III). Two fragments of *p1* were amplified covering nt 553–1056 (no. I) and nt 3295–4530 (no. II). The major part of *p116* was amplified covering nt 1–2883. The primers that were used to generate recombinant proteins were designed with histidine-tags: forward primer, 5'-GAC GACGACAAGATX-insert specific sequence-3'; reverse primer, 5'-GAGGAGAAGCCCGGT-insert specific sequence-3'. All the primers were purchased from DNA Technology APS, Aarhus, Denmark, and are shown in Table 1.

High Fidelity PCR was performed as described previously [29]. The PCR products were annealed to the pET-30 Ek/LIC vector with the Ligation Independent Cloning (LIC) kit (Novagen, Madison, USA) as prescribed by the manufacturer. The recombinant plasmids were expressed in *Escherichia coli* NovaBlue Singles Competent Cells and subsequently purified by

Table 1. List of primers used to amplify DNA fragments for the production of recombinant proteins

Name*	Position (bp)	Sequence 5' to 3'
f-mgpa [†]	1–26	ATGCACCAACCAAAGAAAAGACTGGC
f-mgpa-I	229–257	TCACTAAAGATTACTGGAGAGAACCAGG
r-mgpa-I	945–969	TGGTTTTTCAGGCCTCAGTAAACTCC
f-mgpa-II	2488–2506	ACAGGAGCAGGGTATCGCG
r-mgpa-II	3001–3027	TACATCCTGACTAGTTCAGTTTAACCC
f-mgpa-III	3223–3248	CCTAAATCACTGTGAGATCCAACCTGC
r-mgpa-III [†]	4307–4335	TTATTGTTTTACTGGAGGTTTTGGTGGGG
f-p1 [†]	232–248	TCCCTCCCCCTCACGGG
r-p1 [†]	4868–4883	CACTGGCTTAGGAGCC
f-p1-I	553–568	CCCGTCGA GGTGGCGC
r-p1-I	1042–1056	GGGGGTGGGGGAACC
f-p1-II	3295–3316	ACGCCCCAGGACGTCGGTAACC
r-p1-II	4514–4530	TTGGGGACCT TGA CTGG
f-p116	1–25	ATGAAGCTTAGTGCTATTATCTCCC
r-p116	2863–2883	GTCAATAGGAATACCCAAGCG

All primers were purchased from DNA technology ApS, Aarhus, Denmark.

*The first letter of the primer name denotes the direction of the primer: f, forward; r, reverse.

[†]Primers used for nested PCR.

alkaline lysis as described by Sambrook *et al.* [34]. The purified plasmid vectors were transformed into *E. coli* BL21(DE3) competent cells. For expression of recombinant proteins, *E. coli* BL21(DE3) were induced with 0.5 mM *iso*-propyl-thio-galactoside (IPTG) for 2.5 h at 37°C [34]. The recombinant proteins were purified with HiTrap™ affinity columns (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The recombinant proteins were denoted rMgPa-I,

rMgPa-II, rMgPa-III, rP1-I, rP1-II and rP116 and are schematically outlined in Fig. 1.

Production of rabbit polyclonal antibodies (Pabs)

Rabbit Pabs were produced as described previously [35]. Pab was produced against whole cells of *M. genitalium* G37 (denoted PabG37). Pab against whole cells of *M. pneumoniae* FH (denoted PabFH) was purchased from

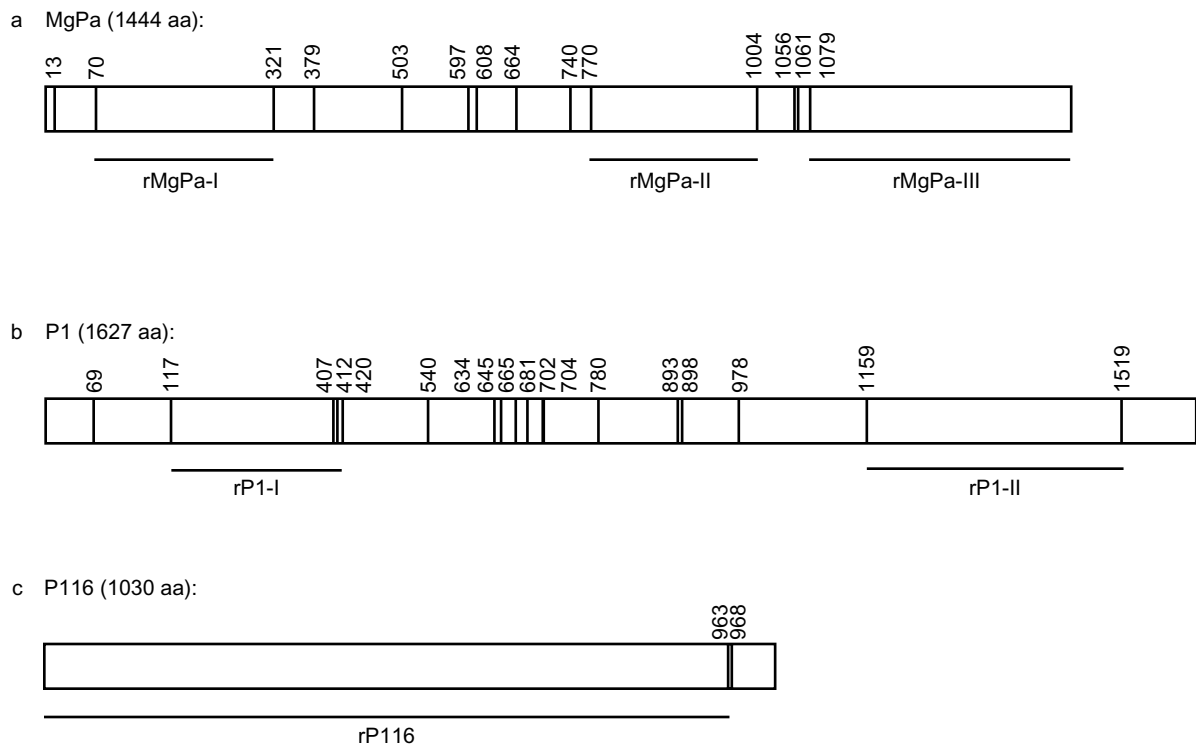


Fig. 1. Schematic illustration of (a) MgPa, (b) P1 and (c) P116 amino acid (aa) sequences showing the position of tryptophan encoded by the UGA stop-codon (black vertical lines). The locations of the recombinant proteins used for the production of monospecific antibodies are shown below each aa sequence (black horizontal lines).

the WHO Mycoplasma Reference Center, Aarhus University, Denmark. Rabbit monospecific antibodies were produced against recombinant proteins as described previously [29]. The sera were denoted Pab() with the name of the recombinant protein in brackets: Pab(rMgPa-I), Pab(rMgPa-II), Pab(rMgPa-III), Pab(rP1-I), Pab(rP1-II) and Pab(rP116).

SDS-PAGE and immunoblotting

Protein (100 μg) of *M. genitalium* or *M. pneumoniae* whole cells or recombinant proteins (50 μg) were dissolved in 150 μg of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, glycerol 10% v/v, SDS 2.3% w/v, β -mercaptoethanol 5% v/v, bromphenol blue 0.05% w/v) and boiled for 2 min. The proteins were separated by SDS-PAGE in 7.5% or SDS-polyacrylamide 10% gels containing a 5% stacking gel.

The proteins were transferred to nitrocellulose (Schleicher & Schull, Dassel, Germany) by electroblotting. The marker was cut from the membrane and stained with Amido Schwartz for 2 min. The rest of the membrane was blocked with blocking buffer (20 mM Tris-base, 500 mM NaCl, gelatine 3%) for 15 min at 37°C.

The immunostaining was performed by a method of BioRad. The membrane was cut into 3-mm strips and incubated with primary antibodies for 1 h at 37°C. The rabbit sera were diluted 1 in 1000 in antibody buffer (20 mM Tris-base, 500 mM NaCl, gelatine 3%, Tween-20 0.05%). The secondary antibody used was alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (H+L) diluted 1 in 3000 and incubated with strips for another hour. The membrane was washed with washing buffer (20 mM Tris-base, 500 mM NaCl, Tween-20 0.05%) after each incubation. Finally, the strips were developed for 10 min with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate p-toluidium (NBT/BCIP) solution.

Preparation of HEp-2 cells for infection with mycoplasma

The human cell line, HEp-2 (ATCC, MD, USA), was cultured in TTP tissue-culture flasks (Medi Cult, Copenhagen, Denmark) containing RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) with 25 mM HEPES-buffer (0.01 M N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid, 0.15 M NaCl, pH 7.2), sodium bicarbonate, fetal calf serum 10%, gentamicin mg/L and 2 mM glutamine, pH 7.2.

The HEp-2 cells were transferred to 24-well Multi-dish plates (Nunc, Roskilde, Denmark) with sterile glass coverslips by loosening the cells with PBS containing trypsin 0.25% and EDTA 0.02%. The cell concentration was adjusted to 50 000 HEp-2 cells/ml of 1640 RPMI medium with penicillin (100 U/ml) 0.05% and

1 ml of the cell suspension was added per well. The plates were incubated overnight in air with CO₂ 5% at 37°C.

Preparation of mycoplasma for infection of HEp-2 cells

M. genitalium and *M. pneumoniae* were cultured in 10-ml tissue-culture flasks as described previously [29]. The mycoplasmas were scraped off in 10–12 ml of RPMI with penicillin (100 U/ml) 0.05% ready for infection of the HEp-2 cells. To avoid the noticeable self-aggregating feature of *M. pneumoniae*, the suspension was sheared through a 27G needle.

Adhesion detection assay

HEp-2 cells, in RPMI with penicillin (750 μl /well), were infected with the mycoplasma RPMI-suspension (50 μl /well) and incubated for 2 h. The infected HEp-2 cells were fixed in methanol 100% at 4°C (0.5 ml/well) for 1 min and washed with PBS. To detect the adhering mycoplasmas, primary Pabs were added for 30 min at 37°C (PabG37 and PabFH were diluted 1 in 2000 and 1 in 1000 respectively, the monospecific antibodies were diluted 1 in 500). A solution (300 μl) of secondary fluorescein isothiocyanate (FITC)-conjugated 'Affini-pure' goat anti-rabbit (GaR) IgG (H+L) (Jackson Immuno Research Laboratories, Pennsylvania, USA) diluted 1 in 100 (FITC-conjugated GaR IgG) and Evans Blue diluted 1 in 10 was added to each well for 30 min at 37°C. The cells were washed twice in PBS before and after the addition of antibodies.

Surface exposure assay

To detect surface proteins of the mycoplasmas, the primary antibodies were added before methanol fixation. Otherwise, the procedure was the same as described for the adhesion detection assay.

Adhesion inhibition assay

The mycoplasma suspension (50 μl) was pre-incubated for 30 min at 37°C with 50 μl of different antibody solutions (1 in 50, 1 in 100, 1 in 200 and 1 in 400) before incubation of the HEp-2 cells. The mycoplasma-antibody suspension (100 μl) was then added to the HEp-2 cells together with 700 μl of RPMI with penicillin and incubated overnight in air with CO₂ 5% at 37°C. Fixation and addition of secondary antibodies were as described in the adhesion detection assay.

Indirect immunofluorescence microscopy (IFM)

Samples prepared for the adhesion detection, surface exposure and inhibition assays were investigated by IFM. A drop of anti-fade solution (p-phenyldiamine dihydrochloride 1 μg /ml in PBS 10% and glycerol

90%, pH 9.0) was placed between the glass coverslips and the slides. Fluorescence microscopy was performed with a Leitz DMR fluorescence microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany).

Results

Recombinant proteins

Recombinant proteins were produced to investigate the adhesion domains of MgPa of *M. genitalium* and P1 and P116 of *M. pneumoniae*. Because the UGA tryptophan codon will create a stop codon in *E. coli*, the regions cloned were limited. Three regions of MgPa were cloned located in the N-terminal, the middle and the C-terminal part (denoted rMgPa nos I–III; Fig. 1a). Two regions of P1 covering the N- and the C-terminal part were cloned (denoted rP1 nos I–II; Fig. 1b). Almost the entire P116 was produced as one recombinant protein because the only two tryptophan UGA codons present are located in the C-terminal part (denoted rP116; Fig. 1c).

SDS-PAGE and immunoblotting of recombinant proteins

The purified recombinant proteins were separated by gel electrophoresis and stained with Coomassie Brilliant Blue (CBB) or transferred to nitrocellulose for immunoblotting. SDS-PAGE of rMgPa (nos I–III) is shown in Fig. 2a (lanes denoted CBB). In immunoblotting with rMgPa (nos I–III) (Fig. 2a, lanes denoted IMB) polyclonal antibody against whole cells of *M. genitalium*, Pab(G37), did not react with rMgPa-I, but reacted moderately with rMgPa-II and strongly with rMgPa-III.

SDS-PAGE of rP1 (nos I–II) and rP116 is shown in Fig. 2b (lanes CBB). In immunoblotting with rP1 (nos I and II) and rP116 (Fig. 2b, lanes IMB) polyclonal antibody against whole cells of *M. pneumoniae*, Pab (FH), did not react with rP1 but reacted strongly with rP1-II and rP116.

Monospecific Pab

Rabbits were immunised with the purified recombinant proteins for the production of monospecific antibodies. In immunoblotting with whole-cell proteins of *M. genitalium* Pab(rMgPa) nos I–III and all the monospecific antibodies reacted strongly with MgPa (Fig. 2c). Correspondingly, in immunoblotting with whole-cell proteins of *M. pneumoniae* Pab(rP1) nos I and II and Pab(rP116), the Pabs reacted strongly with P1 and P116, respectively (Fig. 2d).

Indirect immunofluorescence microscopy (IFM)

IFM was performed to analyse (i) the suitability of the monospecific antibodies for detecting the mycoplasmas

adhering to the HEp-2 cells (adhesion detection assay), (ii) the reaction of the monospecific antibodies with the surface of the mycoplasmas (surface exposure assay) and (iii) the ability of the monospecific antibodies to inhibit cytoadsorption (adhesion inhibition assay). HEp-2 cells, derived from a laryngeal carcinoma, were used as host epithelial cells in the adhesion and the inhibition assays.

Adhesion detection assay

HEp-2 cells, infected either with *M. genitalium* or *M. pneumoniae*, were methanol-fixed before exposing them to the primary antibodies. Methanol is a strong fixative that denatures most proteins and removes lipids in the cell membrane, thus allowing antibodies to penetrate the mycoplasma cells. The bound primary antibodies were detected with an FITC-conjugated goat anti-rabbit immunoglobulin (IgG).

Monospecific Pab(rMgPa) nos I–III were all able to identify *M. genitalium* microcolonies attached to the HEp-2 cells, as visualised by the green fluorescence spots scattered all over the surface of the HEp-2 cells (Fig. 3a–c). However, Pab(rMgPa-II) did not react as strongly as the other two monospecific antibodies (Fig. 3b). Polyclonal antibody against whole cells of *M. genitalium*, Pab(G37), strongly detected cytoadhering mycoplasmas on the HEp-2 cells and served as a positive control (Fig. 3d).

The monospecific antibodies Pab(rP1) nos I and II and Pab(rP116) were all able to identify *M. pneumoniae* cells bound to the HEp-2 cells (Fig. 3e–g). Large green fluorescent spots were identified, showing that the cells of *M. pneumoniae* were clustered on the HEp-2 cells. A polyclonal antibody against whole cells of *M. pneumoniae*, Pab(FH), strongly detected the cytoadsorbed cells of *M. pneumoniae* and served as a positive control (Fig. 3h). In general, the cells of *M. pneumoniae* tended to form micro-colonies rather than adhering as single cells. *M. genitalium* also produced minute colonies but they were smaller than the colonies of *M. pneumoniae*.

Surface exposure assay

To detect the accessibility of the antibodies on the surface of the cytoadhering mycoplasmas, the primary detecting antibodies were added before fixation with methanol. Again, the bound primary antibodies were visualised by FITC-conjugated IgG.

The monospecific Pab(rMgPa-I) and Pab(rMgPa-II) were not able to detect the attached *M. genitalium* cells on the HEp-2 cells (Fig. 3i and j), whereas both Pab(rMgPa-III) and Pab(G37) (Fig. 3k and l) revealed cytoadhering mycoplasmas visualised as green fluorescence spots dispersed on the HEp-2 cells. Likewise, no fluorescence was observed when the monospecific Pab(P1-I) was

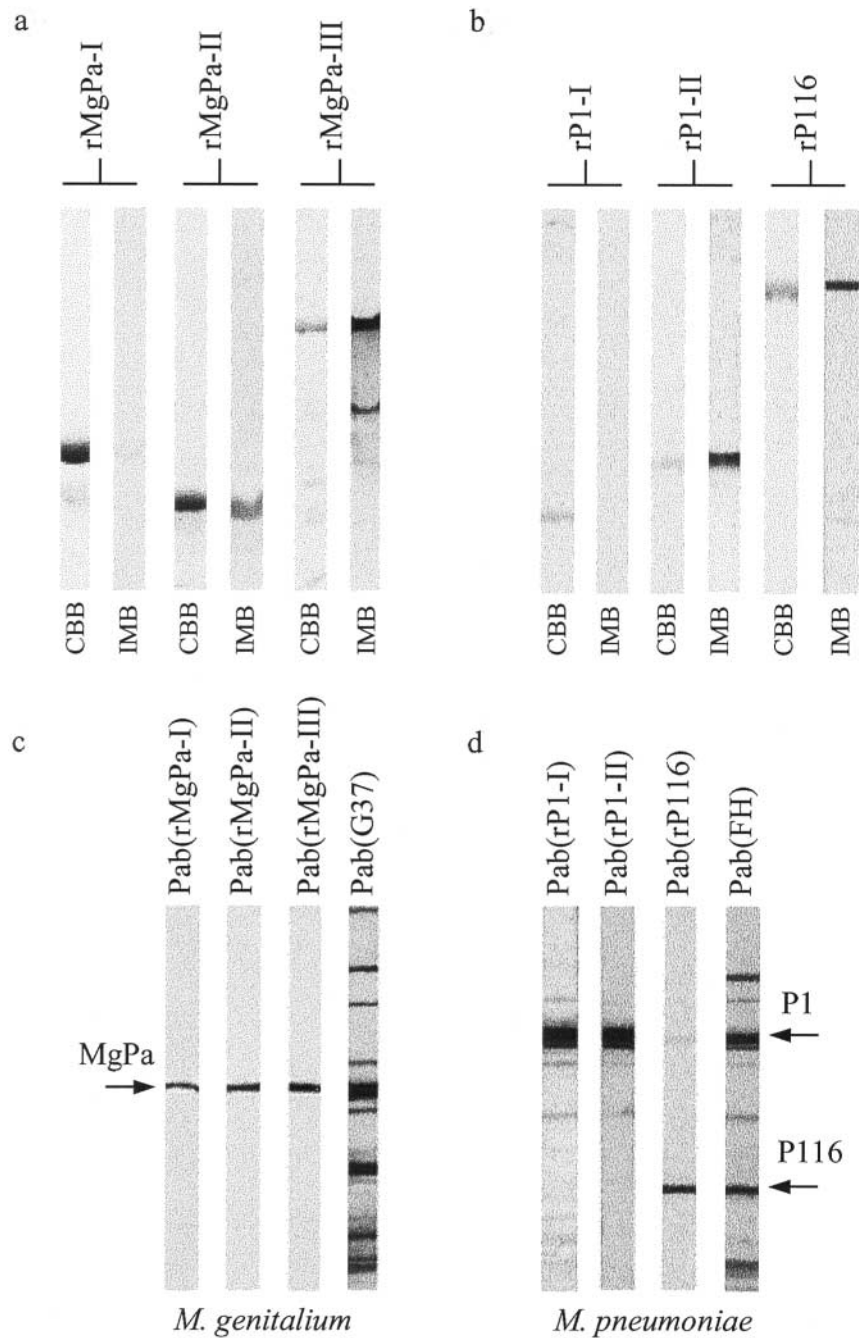


Fig. 2. SDS-PAGE of (a) recombinant MgPa of *M. genitalium* stained with Coomassie Brilliant Blue (lane CCB) and reacted with Pab(G37) in immunoblotting (lane IMB). Note that Pab(G37) did not react with rMgPa-I, reacted intermediately with rMgPa-II and strongly with rMgPa-III. (The faster running protein band (lane rMgPa-III, IMB) is a degradation product of rMgPa-III.) (b) Recombinant proteins, P1 and P116 of *M. pneumoniae* stained with CCB and Pab(FH) in IMB. Note that Pab(FH) did not react with P1-I and reacted strongly with P1-II and P116. Immunoblotting of whole cells of (c) *M. genitalium* reacted with monospecific antibodies directed against N-terminal, middle and C-terminal part of MgPa. All antibodies reacted intensely with MgPa. Pab(G37) directed against whole cells of *M. genitalium* served as a control. (d) *M. pneumoniae* reacted with monospecific antibodies directed against the N- and C-terminal part of P1 and P116. All antibodies reacted strongly with P1 and P116, respectively. Pab(FH) directed against whole cells of *M. pneumoniae* is included as a control.

added to the HEp-2 cells infected with *M. pneumoniae* (Fig. 3m), whereas Pab(P1-II), Pab(P116) and Pab(FH) were all able to detect *M. pneumoniae* cells attached to the HEp-2 cells (Fig. 3n-p).

Negative controls included in the adhesion detection and surface exposure assay were cells of *M. genitalium* and *M. pneumoniae* adhering to HEp-2 cells without

antibodies (Fig. 3q, Table 2) and antibodies without mycoplasmas (Fig. 3r, Table 2) to ensure that non-specific binding of the antibodies did not occur.

Adhesion inhibition assay

The adhesion inhibition assay examined whether the monospecific antibodies were able to block either *M.*

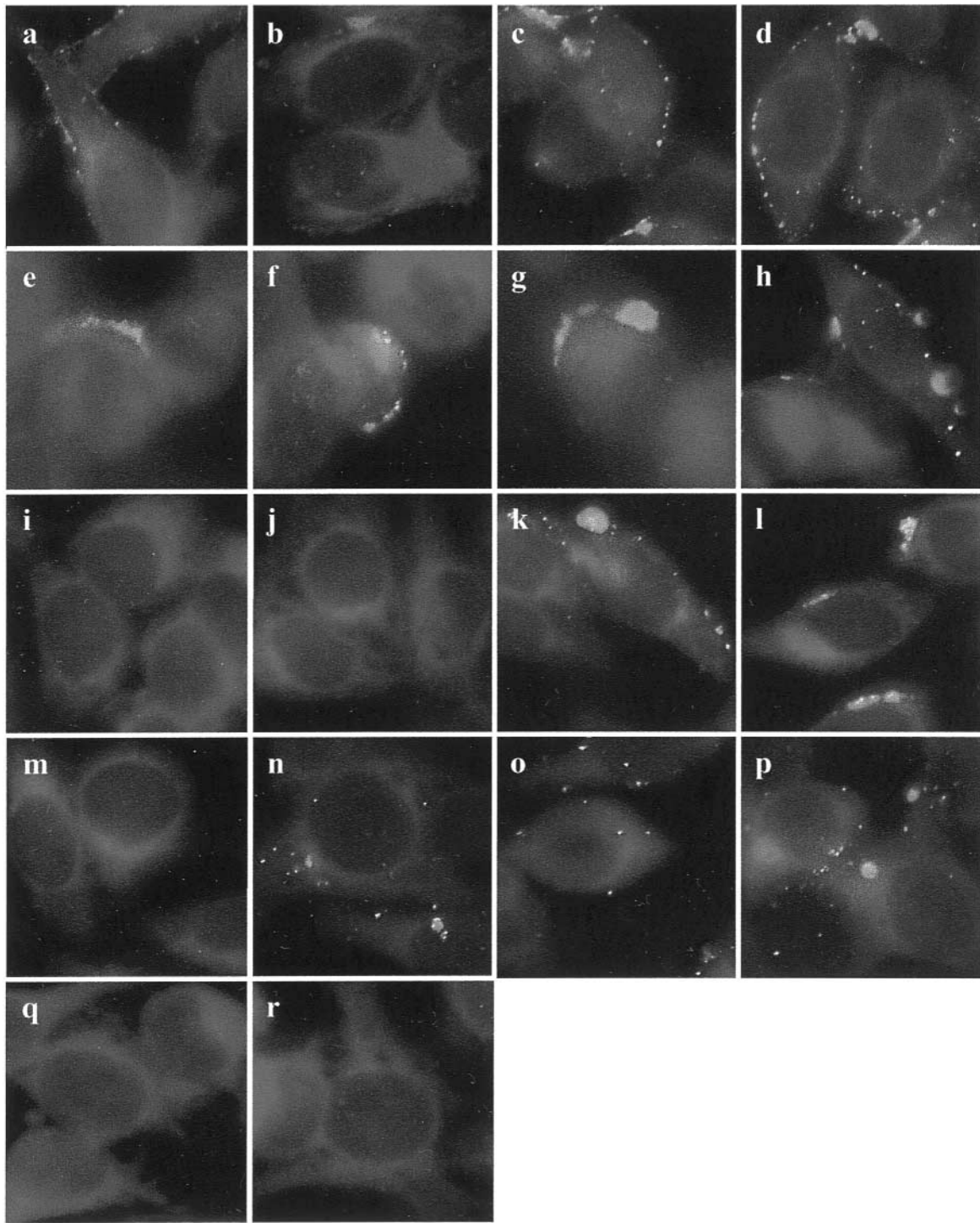


Fig. 3. IFM adhesion detection assay of *M. genitalium* (a–d) and *M. pneumoniae* (e–h). The mycoplasmas attached to the HEp-2 cells were detected by either polyspecific or monospecific antibodies. The detecting antibodies were added after fixation with methanol: (a) Pab(rMgPa-I), (b) Pab(rMgPa-II), (c) Pab(rMgPa-III), (d) Pab(G37), (e) Pab(rP1-I), (f) Pab(rP1-II), (g) Pab(rP116) and (h) Pab(FH). IFM surface exposure assay of *M. genitalium* (i–l) and *M. pneumoniae* (m–p). (i) Pab(rMgPa-I). In this assay the detecting antibodies were added before the methanol fixation: (j) Pab(rMgPa-II), (k) Pab(rMgPa-III), (l) Pab(G37), (m) Pab(rP1-I), (n) Pab(rP1-II), (o) Pab(rP116) and (p) Pab(FH). The negative controls were: (q) mycoplasmas alone (no Pabs) and (r) Pabs alone (no mycoplasmas). Bar, 10 μ m.

genitalium or *M. pneumoniae* from binding to the HEp-2 cells. Dilution series (1 in 50, 1 in 100, 1 in 200 or 1 in 400) of monospecific antibodies were incubated with the mycoplasmas before infection of the HEp-2 cells to test their adherence inhibitory effect. The cytoadhering mycoplasmas were detected with polyclo-

nal antibodies against whole cells of *M. genitalium*, Pab(G37), and *M. pneumoniae*, Pab(FH), rather than the weaker binding monospecific antibodies (see the adhesion detection assay). Again, the mycoplasmas attached to the HEp-2 cells were visualised by the secondary FITC-conjugated goat-anti rabbit IgG.

Table 2. Adhesion of *M. genitalium* and *M. pneumoniae* to HEp-2 cells measured by IFM

Pab	Adhesion detection assay	Surface exposure assay
<i>M. genitalium</i>		
rMgPa-I	+	-
rMgPa-II	+	-
rMgPa-III	+	+
G37	+	+
<i>M. pneumoniae</i>		
rP1-I	+	-
rP1-II	+	+
rP116	+	+
FH	+	+
Negative controls		
Pab (no mycoplasma)*	-	-
Mycoplasma (no Pab)	-	-

*Pabs include: Pab(rMgPa-I), Pab(rMgPa-II), Pab(rMgPa-III), Pab(rP1-I), Pab(rP1-II) and Pab(rP116).

The polyspecific antibodies, Pab(G37) and Pab(FH), were included in the adhesion inhibition assay as inhibiting antibodies because they were expected to reduce the numbers of cytoadhering *M. genitalium* and *M. pneumoniae*. Addition of Pab(G37) (Fig. 4a–d and Fig. 5a4) and Pab(FH) (Fig. 4e–h and Fig. 5b5) also showed that mycoplasmas were inhibited from binding to the HEp-2 cells by increasing the antibody concentration. This correlation decreased nearly exponentially with almost no inhibition with the minimum antibody concentration (1 in 400) and only 5% of *M. genitalium* and 9% of *M. pneumoniae* adhering to the HEp-2 cells at the maximal concentration of antibody (1 in 50) compared with positive controls (Fig. 4a–h, Table 3). Mycoplasma-infected HEp-2 cells without antibody and with pre-immune serum served as positive controls (Fig. 4y, Table 3) (adhesion detection assay).

Similarly, the adhesion inhibition assay was performed with monospecific antibodies against different parts of MgPa of *M. genitalium* (Pab(rMgPa) nos I–III). No inhibition of cytoadsorption of *M. genitalium* was observed with either Pab(rMgPa-I) or Pab(rMgPa-II) because numerous cells of *M. genitalium* (86% and 61%, respectively) were seen on the HEp-2 cells when the 1 in 50 dilution was used (Fig. 4u and v, Fig. 5a1 and 2). In contrast, Pab(rMgPa-III) was shown to inhibit the binding of *M. genitalium* to the HEp-2 cells as seen in Fig. 4i–l, showing the dilution series. Pab(rMgPa-III) inhibited the cytoadsorption of *M. genitalium* just as well as the polyspecific Pab(G37) as seen in the exponential decreasing plots (Fig. 5a3 and 4). The cytoadherence of *M. genitalium* was reduced to c. 3%, when Pab(rMgPa-III) was diluted 1 in 50 (Table 3).

No inhibition of adhesion of *M. pneumoniae* to HEp-2 cells was observed with Pab(rP1-I) (Fig. 4x), as no decrease in the number of attached cells of *M. pneumoniae* was observed when the antibody concen-

tration was increased. When the maximal antibody concentration was used for inhibition, 94% of the micro-colonies were still attached to the HEp-2 cells (Fig. 5b1 and Table 3). In contrast, Pab(rP1-II) and Pab(rP116) both inhibited the adsorption of *M. pneumoniae* to the HEp-2 cells as seen in Fig. 4m–p and Fig. 4q–t showing the dilution series. Pab(rP116) inhibited *M. pneumoniae* to the same extent as Pab(rP1-II) (Fig. 5b2 and 3) but, when the antibodies were added together, it seemed that the inhibitory effect of these was increased. The plot of inhibition caused by a mixture of Pab(rP1-II) and Pab(rP116) is shown in Fig. 5b4 and appeared very similar to the plot of Pab(FH) (Fig. 5b5). The average number of attached *M. pneumoniae* micro-colonies at the maximal antibody dilution (1 in 50) of mixed anti-P1 and anti-P116 was reduced to 6% of that of the positive controls (Table 3). The reduction of attached *M. pneumoniae* was slightly lower when Pab(rP1-II) and Pab(rP116) were used alone, with the higher percentages of 15% and 10% (Fig. 5b2 and 3, Table 3).

Discussion

This study shows evidence for the surface exposure of MgPa of *M. genitalium* and P1 and P116 of *M. pneumoniae* as well as the involvement of the proteins in adhesion to host epithelial cells. The surface exposure of MgPa, P1 and P116 has been determined in several studies with trypsinization [22, 27, 36]. Failure of adherence to tracheal explants after protease treatment following re-attachment when P1 was regenerated demonstrated P1 as an attachment factor. The P1 protein is considered to be the major ligand mediating attachment of virulent *M. pneumoniae* to the host cell membrane and clustering of P1 at the tip-organelle is decisive for a successful adhesion [19]. An earlier study showed that pre-treatment of *M. pneumoniae* with antiserum directed against P1 blocked cytoadherence to hamster tracheal rings by up to 80% [37]. Electron microscopy with monoclonal antibodies (MAbs) produced against MgPa of *M. genitalium* revealed that the protein is located mainly in the tip structure, which suggested MgPa to be the counterpart of P1 [27].

It was shown by IFM that, of the protein regions investigated in this study, only the C-terminal part of MgPa (aa 1075–1444) and the C-terminal part of P1 (aa 1107–1518) are exposed on the surface of *M. genitalium* and *M. pneumoniae*. These results were consistent with the adhesion inhibition experiments showing that blocking of the C-terminal part of MgPa of *M. genitalium* and the C-terminal part of P1 of *M. pneumoniae* by monospecific antibodies inhibited the mycoplasmas from binding to HEp-2 cells. This indicates that the C-terminal part of both MgPa and P1 is involved in cytoadhesion. However, the possibility that steric hindrance by the monospecific antibody bound to the C-terminal part might cause the inhibition

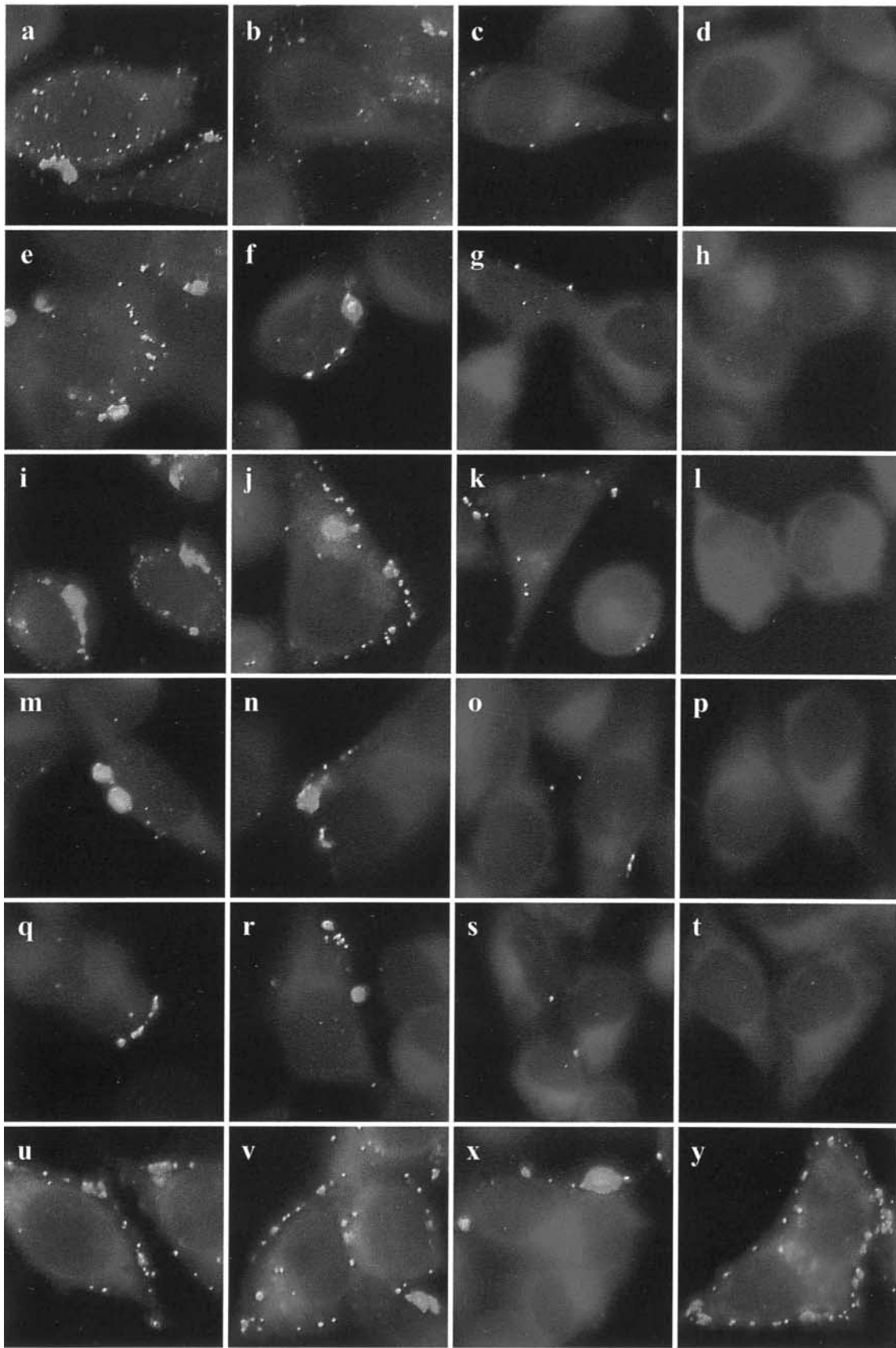


Fig. 4. IFM adhesion inhibition assay. *M. genitalium* and *M. pneumoniae* were pre-incubated with either polyspecific or monospecific antibodies in different dilutions (1 in 50, 1 in 100, 1 in 200, 1 in 400) before infection of the HEp-2 cells. These antibodies were: (a–d) Pab(G37), (e–h) Pab(FH), (i–l) Pab(rMgPa-III), (m–p) Pab(rP1-II), (q–t) Pab(rP116), (u) Pab(rMgPa-I) diluted 1 in 50, (v) Pab(rMgPa-II) diluted 1 in 50, (x) Pab(rP1-I) diluted 1 in 50 and (y) pre-immune serum diluted 1 in 50. The primary antibodies in the adhesion inhibition assay were Pab(G37) (1 in 2000) and Pab(FH) (1 in 1000) for detection of *M. genitalium* and *M. pneumoniae*, respectively. Bar, 10 µm.

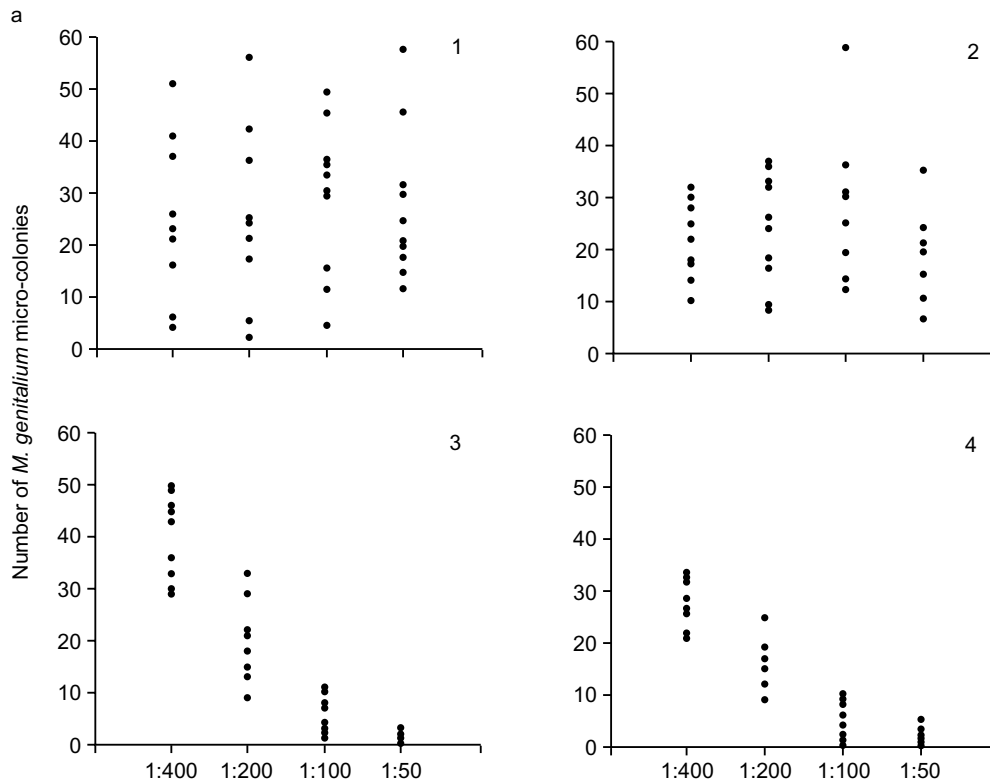


Fig. 5. Results of the IMF adhesion inhibition assay. The number of micro-colonies of cytoadhering mycoplasmas is plotted against the dilutions of potential inhibiting antibodies. (a) Plots of the attached *M. genitalium* micro-colonies for each antibody: Pab(rMgPa) nos I (1), II (2) and III (3) and Pab(G37) (4). Note that Pab(rMgPa-III) and Pab(G37) reduced the number of colonies when the antibody concentration increased. (b) Adherent micro-colonies of *M. pneumoniae* with Pab(rP1) nos I (1) and II (2), Pab(rP116) (3), the combination of Pab(rP1-II) and Pab(rP116) (4) and Pab(FH) (5).

cannot be excluded. Nevertheless, it can be concluded that the adherence domain is located near the C-terminal part of both proteins.

Monospecific antibodies against the N-terminal, Pab(rMgPa-I), covering aa 77–320 and the middle part Pab(rMgPa-II); aa 830–1009, of MgPa did not inhibit *M. genitalium* from binding to the HEp-2 cells. Neither did the monospecific antibody against the N-terminal part of P1, Pab(rP1-I); aa 185–357, block *M. pneumoniae* from adhering to the HEp-2 cells. This result was in agreement with the non-surface exposure of the N-terminal and the middle parts of MgPa and the N-terminal part of P1 as shown by IFM. Furthermore, immunoblotting of N-terminal recombinant proteins (rMgPa-I and rP1-I) was non-reactive with polyspecific antibodies directed against whole mycoplasma cells, Pab(G37) and Pab(FH). This indicates that the N-terminal part is not exposed on the surface and, therefore, antibodies are not generated to this part during immunisation. The middle part of MgPa showed a weak reaction in immunoblotting.

The finding of an attachment epitope located in the C-terminal part of P1 of *M. pneumoniae* (aa 1107–1518) in this study was consistent with the observation by Dallo *et al.* [38]. They showed a C-terminal region of 13 amino acids involved in cytoadhesion (aa 1382–1394) which is also located in the C-terminal region of

P1 (rP1-II). A C-terminal attachment epitope was also found in the studies of Opitz and Jacobs [39] and Gerstenecker and Jacobs [40] but an adherence region sited only in the C-terminal part was not in agreement with their results. They showed adhesin epitopes to be present in several positions of MgPa of *M. genitalium* and P1 of *M. pneumoniae*.

In the studies of Opitz and Jacobs [39] and Gerstenecker and Jacobs [40], indirect adhesion inhibition assay with inhibiting MAbs against MgPa of *M. genitalium* and P1 of *M. pneumoniae* was used. For epitope scanning, the MAbs were used with overlapping octapeptides in ELISA to detect the attachment epitopes. The methods used in these studies are different from those used in the present study and may, therefore, explain the different results.

Interestingly, the adhesion domain found in the C-terminal part of both MgPa and P1 has also been determined to be immunodominant in other studies. Recently, we have shown that the most immunogenic region of MgPa is located in the aa region 1248–1364 [29] in agreement with observations by other groups who have determined the immunodominant regions of P1 to cover aa 1382–1394 [38] and aa 1124–1131 [41], which are both included in rP1-II. Thus, it seems that the adherence and immunogenic epitopes are predominantly located in the C-terminal part of MgPa and P1.

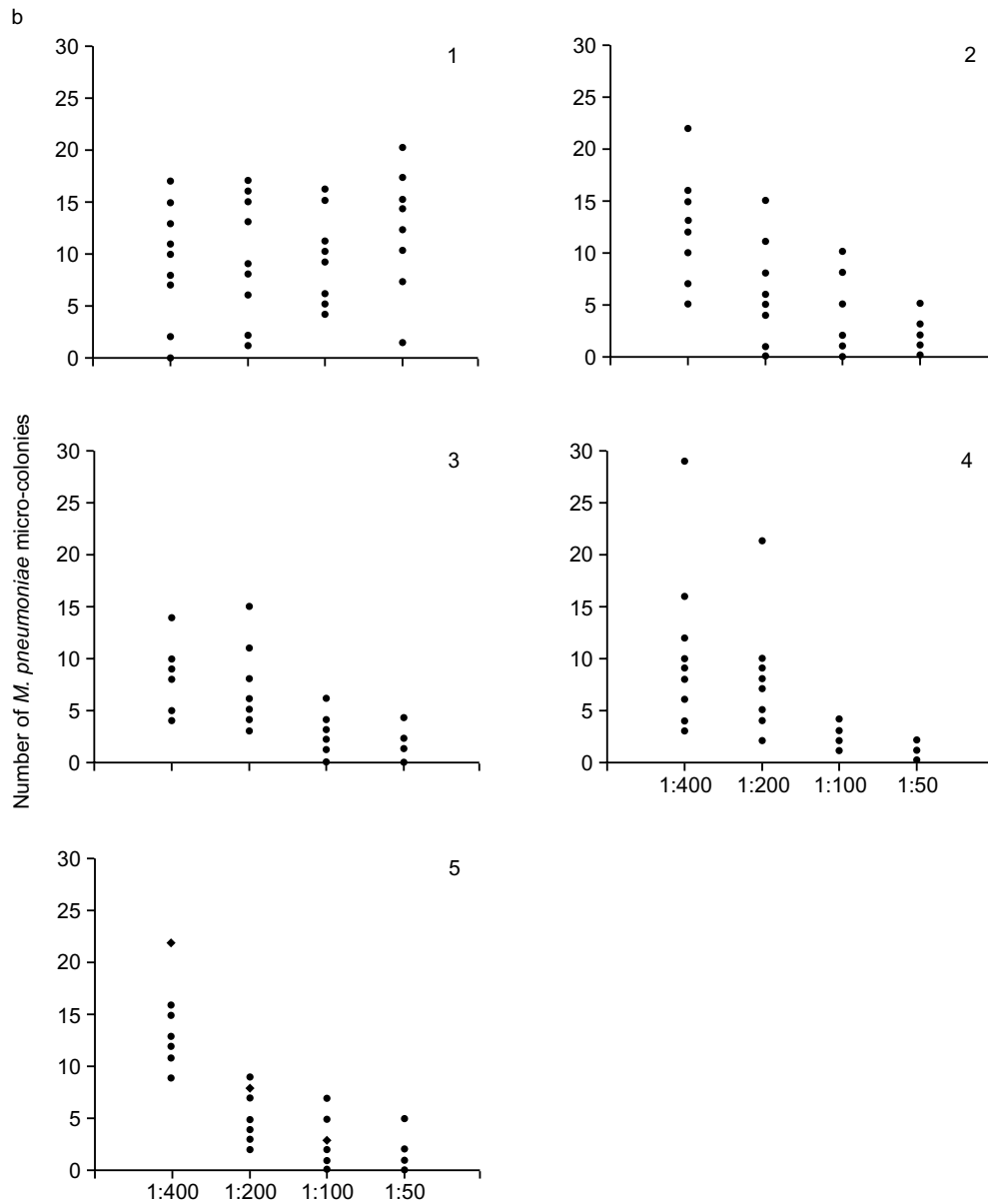


Fig. 5. (continued).

No previous reports have shown whether *M. pneumoniae* protein P116 is implicated in adhesion to host epithelial cells. The location of the 116-kDa protein to the surface of *M. pneumoniae* observed by IFM (this study) and by trypsin degradation [22] was in agreement with the immunofluorescence adhesion inhibition results showing that Pab(rP116) was able to block the adherence of *M. pneumoniae* to HEp-2 cells. This implies that the protein P116 of *M. pneumoniae* is an adhesin implicated in the cytoadherence of *M. pneumoniae*, like the two documented adhesins P1 [36] and P30 [27].

Krause and Baseman [37] analysed the adherence of *M. pneumoniae* proteins to hamster tracheal epithelial cells. They found that radiolabelled *M. pneumoniae* protein of 110 kDa, which they denoted P2, was able to bind to the hamster tracheal cells, and the adhesion was competitively inhibited by unlabelled P2 protein, indi-

ating a possible role for this protein as an adhesin to a specific host receptor. The P116 (116-kDa protein) of *M. pneumoniae* analysed in this work could be the equivalent of the P2 protein described by Hu *et al.* [36] and Krause and Baseman [37] because the proteins are similar in both size and strong immunogenicity.

Cross-linking studies have shown that the P1 adhesin is complexed to other proteins involved in cytoadhesion in the cell membrane of *M. pneumoniae* [23, 24]. However, the 116-kDa protein was not found to be a part of the P1 multi-protein complex at the tip structure of *M. pneumoniae*. The definite location of P116 in the cell membrane in proportion to P1 or the P1 multi-protein complex remains to be clarified. Furthermore, it is not known whether P1 and P116 are interdependent in the adherence of *M. pneumoniae* to host epithelial cells. However, the results of the adhesion inhibition assay showed a higher degree of inhibition when both

Table 3. The number of *M. genitalium* and *M. pneumoniae* micro-colonies per HEp-2 cell in different solutions of Pab.

Inhibiting Pab	Number of micro-colonies/HEp-2 cell (percent of positive control) at antibody dilution			
	1 in 400	1 in 200	1 in 100	1 in 50
<i>M. genitalium</i>				
rMgPa-I	26.2 (85)	26.4 (85)	28.7 (93)	26.7 (86)
rMgPa-II	21.0 (68)	23.9 (77)	28.2 (91)	18.8 (61)
rMgPa-III	39.4 (127)	18.8 (61)	5.6 (18)	0.8 (3)
G37	28.0 (91)	20.0 (65)	4.1 (13)	1.5 (5)
Pre-immune sera	ND	ND	ND	30.9
<i>M. pneumoniae</i>				
rP1-I	9.6 (73)	9.5 (73)	9.3 (71)	12.3 (94)
rP1-II	12.9 (99)	7.0 (53)	3.0 (23)	2.0 (15)
rP116	9.6 (73)	7.2 (55)	2.1 (16)	1.3 (10)
rP1-II and rP116	10.9 (83)	8.2 (63)	2.4 (18)	0.8 (6)
FH	13.8 (105)	5.9 (45)	2.2 (17)	1.2 (9)
Pre-immune sera	ND	ND	ND	13.2

The number of micro-colonies was calculated relative to the positive control from the adhesion detection assay (mycoplasma, no Pabs) and given as the percentage (in brackets). Pab(G37) and Pab(FH) were used as the detecting antibodies for *M. genitalium* and *M. pneumoniae*, respectively.

ND, not determined.

Pab(rP1-II) and Pab(rP116) were added simultaneously. This may indicate that two ligand sites (P1 and P116) are needed for an efficient adhesion of *M. pneumoniae* to host cells.

The high degree of inhibition activity (>90%) of the monospecific antibodies to MgPa of *M. genitalium* and P1 and P116 of *M. pneumoniae* demonstrated that each of the antigens is crucial for cytoadhesion. The attachment of the remaining mycoplasmas could be mediated by accessory adhesion proteins such as P30 of *M. pneumoniae* and the homologous P32 of *M. genitalium*. However, it was expected that the antibodies raised against whole cells of *M. genitalium* and *M. pneumoniae* would completely inhibit the cytoadherence of the mycoplasmas to the HEp-2 cells, but a few mycoplasmas were still attached to the HEp-2 cells at the 1 in 50 antibody dilution. This observation could be due to (i) non-saturating antibody conditions, (ii) the mycoplasmas managing to attach to the HEp-2 cells despite the surface covering antibodies and (iii) avoidance of antibodies because of intra-species variation.

The fact that the antibodies, produced against immunogenic surface proteins in this in-vitro study, reduced the number of attached mycoplasmas suggests a protective function of the humoral immune defence system *in vivo*. Perhaps the specific antibodies produced by the host give protection against a re-infection with *M. genitalium* or *M. pneumoniae* as suggested in many studies with animal models [42–45]. This could explain the greater prevalence of *M. pneumoniae* pneumonia in children and young adults rather than adults and elderly people [46, 47] and is in agreement with the proportional correlation between

increasing age and the presence of antibodies to *M. pneumoniae* [48].

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