Antigenic characterization and cytolocalization of P35, the major *Mycoplasma penetrans* antigen

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***Mycoplasma penetrans* is a mycoplasma with unique morphology, recently identified in urine samples collected from HIV-infected patients. This mycoplasma has been found to be statistically associated with HIV infection, and to be cytopathic *in vitro*. The dominant antigen recognized during natural and experimental infections is an abundant lipoprotein, P35, which, upon extraction, segregates in the Triton X-114 detergent phase. It is used as the basis of *M. penetrans*-specific serological assays. Although mycoplasma lipoproteins, including *M. penetrans* P35, are the main antigens recognized by the host humoral immune response, very little is known about the nature of the epitopes involved. Immunoelectron microscopy revealed that all P35 is exposed at the cell surface and is distributed all over the membrane. P35 linear B-epitopes were mapped by an ELISA approach based on a set of overlapping peptides covering the entire mature polypeptide. The immunoreactivity of the peptides was first tested with sera from immunized animals. The dominant B-epitopes were found at the C- and N-terminal regions, in partial agreement with algorithmic predictions. Patient sera were evaluated with the same assay. Only some reacted with linear epitopes whereas others did not, indicating the importance of P35 nonsequential epitopes. Statistical analysis of the results allowed the definition of a set of peptides which were clearly immunodominant. Finally, the P35-encoding gene was modified by *in vitro* mutagenesis to allow the production and purification of a recombinant protein (rP35Δ0) in *Escherichia coli*. The antigenicity of rP35Δ0 was tested by Western blotting and compared to that of another recombinant product, rP35Δ3, a truncated P35 polypeptide. Although rP35Δ0 reacted with the *M. penetrans*-seropositive patient sera tested, rP35Δ3 was only immunoreactive with one of six sera. This result confirmed that P35-nonsequential epitopes dominate during *M. penetrans* infection. Our results have important implications for the understanding of lipoprotein antigenicity during mycoplasma infections. In addition, the P35-derived immunodominant synthetic peptides defined in this study, as well as the purified rP35Δ0, provide the antigenic material for the necessary improvement of *M. penetrans* serological assays.**

Keywords: epitope mapping, lipoprotein, immunogenicity, *Mycoplasma penetrans*

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Abbreviations: AI, antigenic index; CO₅₀, cut-off 50%; CCU, colour-changing unit; Pam3-CYS, (palmitic acid)₃-Cys-Ser-Lys₄-OH; TEM, transmission electron microscopy.

INTRODUCTION

Mycoplasma penetrans is a novel mollicute species with a unique morphology characterized by an elongated flask shape with a densely packed terminal tip-like structure. It penetrates eukaryotic cells (hence its name), has cytopathic effects *in vitro* (Giron *et al.*, 1996; Lo *et al.*, 1992), and activates peripheral T-lymphocytes from both HIV-seronegative and -seropositive individuals (Sasaki *et al.*, 1995). *M. penetrans* was initially isolated from urine samples from six HIV-infected male homosexuals (Lo *et al.*, 1991a, 1992), and sero-epidemiological studies demonstrated that *M. penetrans* was associated with HIV infection, at least among male homosexual populations (Grau *et al.*, 1995; Wang *et al.*, 1992, 1993). As some mycoplasmas can act in synergy with HIV to induce cell killing *in vitro* (Lemaitre *et al.*, 1992; Lo *et al.*, 1991b), we have suggested that *M. penetrans* may be a cofactor of HIV in viral transmission and/or disease progression (Blanchard, 1997; Blanchard & Montagnier, 1994; Blanchard *et al.*, 1997; Brenner, 1996; Grau *et al.*, 1998). Until recently, *M. penetrans* had only been isolated from HIV-infected patients, and was not associated with any pathology. However, the recent isolation of this mycoplasma from an HIV-seronegative person suffering a primary anti-phospholipid syndrome suggests that *M. penetrans* may, at least under certain circumstances, be pathogenic (A. Yanez and others, unpublished).

As *M. penetrans* isolation from clinical samples is extremely complex and time-consuming (Lo *et al.*, 1991a, 1992), infections with this mycoplasma are almost exclusively detected by serological assays using a Triton X-114 extract of the mycoplasma to detect *M. penetrans*-specific antibodies (Wang *et al.*, 1992). This extract contains two major polypeptides with molecular masses of 35 kDa (P35) and 38 kDa (P38). P35 and P38 are acylated, and we have isolated and sequenced the *p35* gene, which encodes P35 (Ferris *et al.*, 1995). The *p35* sequence encodes a polypeptide with an N-terminal signal sequence with a cleavage site for signal peptidase II. Since some sera containing antibodies against *Mycoplasma pneumoniae* or *Ureaplasma urealyticum* cross-react with P38, it was proposed to restrict the criteria for diagnosing *M. penetrans* seropositivity to a P35-specific reaction in Western blotting (Grau *et al.*, 1995).

The goal of this study was to further characterize P35, the major *M. penetrans* antigen. Its antigenicity was studied. To define sequential epitopes of this lipoprotein, we tested a set of overlapping synthetic peptides by ELISA against different sera, an approach similar to that previously used for other bacterial lipoprotein antigens such as PAc from *Streptococcus mutans* (Matsushita *et al.*, 1994), OspA from *Borrelia burgdorferi* (Ma *et al.*, 1995) and TmpA from *Treponema pallidum* (Antoni *et al.*, 1996). The antigenicity of recombinant P35 and of a truncated P35 was also evaluated. Finally, we investigated the antigenicity of different post-translationally modified P35 polypeptides.

METHODS

Growth conditions and antigen preparation. *M. penetrans* (strain GTU-54-6A1), initially isolated by S.-C. Lo (Armed Forces Institute of Pathology, Bethesda, MD, USA), was kindly provided by J. Tully (National Institute of Allergy and Infectious Diseases, Frederick, MD, USA) and was cultured in SP-4 medium containing 10% (v/v) foetal calf serum (Tully *et al.*, 1977). To determine whether or not P35 is phosphorylated, mycoplasmas were cultured for 24 h at 37 °C in complete SP-4 medium containing $H_3^{32}PO_4$ (Amersham) at 9.25×10^4 Bq (ml culture medium)⁻¹. *M. penetrans* antigens were extracted with the neutral detergent Triton X-114 and amphiphilic polypeptides, which include lipoproteins, were concentrated by detergent-phase partitioning as previously described (Ferris *et al.*, 1995).

Animal and human sera. Two rabbits (1 and 2) and one macaque (9063) were immunized with the Triton X-114 extract from *M. penetrans*, as previously described (Grau *et al.*, 1995). Another rabbit (3) was immunized with a C-terminal synthetic peptide from the P35 (V²⁸⁸KSEKDSTN-NNKYTVTLK³⁰⁵), and the hyperimmune serum was used as a positive control in the epitope-mapping assay (see below). Murine anti-P35 monoclonal antibody (mAb 7, purified IgG1 from ascitic fluid, 300 µg ml⁻¹) was kindly provided by T. Sasaki (National Institute of Health, Tokyo, Japan). In another study conducted in our laboratory, three macaques (91810, 92926 and 8670) were experimentally infected with *M. penetrans* strain GTU-54-6A1. The sera used were those collected when the strongest humoral response against P35 was detected by Western blotting.

The seropositivity of human sera to *M. penetrans* was first tested by ELISA using the detergent phase of the Triton X-114 extraction from *M. penetrans* as antigen. Sera positive in the ELISA were tested by Western blotting: reaction with the P35 polypeptide was required to diagnose *M. penetrans* seropositivity, as previously determined (Grau *et al.*, 1995).

Growth-inhibition assay. To evaluate the ability of the various antibodies to inhibit *M. penetrans* growth, serial 10-fold dilutions of the mycoplasma were cultured in complete SP-4 medium containing the antibody at the dilution indicated. After incubation at 37 °C for 3–4 d, the colour change of the serial dilutions was compared to that of a culture without any antibody. The ability of antibodies to inhibit *M. penetrans* growth was expressed as the logarithm of the difference between the two cultures. Each experiment was performed in duplicate.

Protein quantification, SDS-PAGE and Western blotting. Protein concentrations were determined using the Micro BCA Protein Assay Reagent Kit (Pierce). SDS-PAGE was conducted according to Laemmli (1970) and proteins were stained with Coomassie blue. For Western blotting, the proteins resolved by SDS-PAGE were electro-transferred onto nitrocellulose membranes at 4 mA cm⁻² for 1 h. Membrane-bound polypeptides were immunologically detected as previously described (Ferris *et al.*, 1995). Putative glycosylation with mannose or glucose residues in P35 was evaluated by a Western blotting technique using a peroxidase-conjugated *Canavalia ensiformis* lectin (ConA) as described for the identification of N- and O-glycosylated bacterial proteins such as the 19 kDa antigen from *Mycobacterium tuberculosis* (Garbe *et al.*, 1993; Herrmann *et al.*, 1996).

Transmission electron microscopy (TEM) and immunogold labelling. Immunogold labelling was used on whole cells and

on ultra-thin sections with 0.03 mg mAb 7 ml⁻¹ to locate the P35 polypeptide in the mycoplasma cell. For immunogold labelling of whole mycoplasma cells, the procedure described by Le Gall *et al.* (1997) was followed. The secondary antibodies (goat anti-mouse; Amersham) were conjugated with 10 nm gold particles and the grids were negatively stained with 1% (w/v) phosphotungstic acid. Immunogold labelling of ultra-thin cryosections was as described by Adam *et al.* (1995), except that mycoplasmas were fixed with 4% (w/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde before ultra-thin sectioning with a cryotome, and the detergent Tween 20 was omitted from the washes. The secondary antibodies (goat anti-mouse; Amersham) were conjugated with 5 nm gold particles. The preparations were examined with a 1200 EX electron microscope (JEOL) operating at an accelerating voltage of 80 kV.

ELISA. Microtitre plates were coated with *M. penetrans* Triton X-114 extract as described by Grau *et al.* (1995). In some experiments, the Triton X-114 extract was digested by proteinase K (1 µg per 2.5 µg protein) for 2 h at 56 °C prior to coating as indicated; the disappearance of Coomassie blue-stainable polypeptides resulting from the proteinase K treatment was verified by SDS-PAGE. When Pam₃-CYS ([palmitic acid]₃-Cys-Ser-Lys₄-OH; Boehringer Mannheim) was used as the antigen, it was coated in 0.2% (w/v) TCA for 1 h at 37 °C. For reduction and alkylation of putative disulphide bonds in P35, the Triton X-114 extract was pre-treated with 10 mM DTT and 50 mM iodoacetamide for 30 min at room temperature.

Linear B-epitope mapping. A set of 81 overlapping dodecapeptides (12-mers) covering the complete amino acid sequence of mature P35 was synthesized on polyethylene pins by Chiron Mimotopes as reported by Geysen *et al.* (1987). Each peptide overlapped the preceding one by eight amino acids. The peptides were provided in solution after cleavage from the polyethylene pins. The quality of the synthesis was evaluated by analytical HPLC amino acid analysis of two control peptides, and the serological reactivity of an uncleaved peptide was verified with control serum. To optimize the recognition conditions by sera, the peptides were linked at the C-terminus to a biotin-SGSG arm which allowed peptide binding to streptavidin-coated plates. Lyophilized peptides (1.2 µmol) were resuspended in 200 µl DMSO and kept at -80 °C. Working solutions were obtained by diluting this DMSO stock to 1% (v/v) in PBS, 0.1% (w/v) BSA, 0.01% (w/v) sodium azide, and were stored at 4 °C until use. Streptavidin was coated onto 96-well microplates at 100 ng per well in carbonate/bicarbonate buffer, pH 9.6, for 1 h at 37 °C. Peptide working solutions were diluted 1:10 in PBS, 0.1% BSA and coated onto plates by incubation for 1 h at room temperature. The reactivities of peptides with antibodies were determined by ELISA according to the manufacturer's instructions (Chiron Mimotopes). The bound antibodies were detected with either peroxidase-conjugated goat anti-mouse IgG (heavy and light chains; Bio-Rad) or anti-human IgG (heavy and light chains; Bio-Rad). A peptide was scored as being specifically recognized only if the serum from the same animal obtained prior to the experimental infection did not react. As a positive control, the hyperimmune serum from rabbit 3 was used in ELISA against the appropriate biotinylated peptides.

Epitope prediction and statistical analysis of the data. The method of Jameson & Wolf (1988) was used to predict the antigenicity of the mature P35. The antigenic index (AI) was deduced from the index of hydrophilicity (H), flexibility (F),

surface probability (SP), and the predicted secondary structure of the molecule according to Chou & Fasman [CF; Chou & Fasman (1978)] and Garnier *et al.* [RG; (Garnier *et al.*, 1978)], using the formula: $AI = 0.3H + 0.15SP + 0.15F + 0.2CF + 0.2RG$. A cut-off value of 1.35 was obtained using the formula mean + (4 × SD) on the 166 (50%) lowest AI values.

Data obtained from epitope mapping with human sera were analysed as follows. For each serum, the mean (μ) and the standard deviation (σ) of the 40 lowest values at A₄₉₀ (which correspond to 50% of the data) were calculated and the cut-off 50% (CO₅₀) was calculated as $CO_{50} = \mu + 4\sigma$. An A₄₉₀ was considered positive when higher than CO₅₀. Due to the size of populations, the exact Fisher method was used and calculations were performed using the STATISTICA software (StatSoft). For human sera, a frequency of peptide recognition ≥ 25% was considered significant.

Cloning and mutagenesis of the p35 gene and production of recombinant proteins. The gene encoding the P35 polypeptide lacking the 5' N-terminal signal sequence-encoding fragment was amplified by PCR from the plasmid pMP11 (Ferris *et al.*, 1995) using the following primers: 5' TACGGGATCCTTCTTCTTGTTCTTCTACAAGTGAAAAC 3' and 5' TCGATCCAATTGTCTGCAGGCTAAAAGTTGC 3'. The underlined nucleotides were added for cloning purposes and correspond to *Bam*HI and *Pst*I restriction sites, respectively. PCR amplification was performed with 20 pmol each primer, 25 mM each dNTP, 10 µl 10 × *Taq* DNA polymerase buffer and 2.5 U *Taq* DNA polymerase (Perkin Elmer) in a final volume of 100 µl. The thermal profile was initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, primer annealing at 55 °C for 1 min and extension at 72 °C for 2 min, and finally an incubation at 72 °C for 10 min. The 1 kb amplicon was digested with *Bam*HI and *Pst*I, and ligated to *Bam*HI/*Pst*I-digested pQE31 (Qiagen). In the construct obtained, the p35 gene is under the control of a regulated promoter and the recombinant protein is fused to a His₆ N-terminal tag. The two TGA triplets within the p35 gene were changed to TGG triplets (encoding tryptophan universally) by PCR-mediated mutagenesis (QuickChange Site-Directed Mutagenesis Kit; Stratagene). The mutated plasmid allowing p35 translation in *E. coli* was designated pQEP35Δ0, and encoded a polypeptide with a calculated molecular mass of 37.4 kDa. This plasmid was also subjected to another PCR-mediated mutagenesis using the primer p35mut3 (5' AACAGCAAAAGATGGTACAAACAGATGTTAGTATGG 3') to insert a frame-shift mutation at the underlined position. The plasmid obtained was designated pQEP35Δ3, and encoded a truncated form of P35 (C¹-G¹⁸²) with a calculated molecular mass of 21.6 kDa. The relevant sequences in both pQEP35Δ0 and pQEP35Δ3 were verified by DNA sequencing.

For the expression of recombinant products, each pQE-derived plasmid was used to transform the M15(pREP4) strain of *E. coli* supplied with the QIAexpression Kit (Qiagen). *E. coli* transformants containing either pQEP35Δ0 or pQEP35Δ3 were grown at 37 °C with shaking in 1 l of LB medium supplemented with 25 µg kanamycin ml⁻¹ and 100 µg ampicillin ml⁻¹ to OD_{0.6}. Production of the recombinant proteins was induced by the addition of IPTG to the bacterial culture, to a final concentration of 2 mM, and incubation for 5 h at 37 °C. *E. coli* cells were then recovered by centrifugation at 12000 g for 10 min at 4 °C and the pellets were stored overnight at -20 °C. Bacteria were then thawed and lysed by sonication in 50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0, at 0 °C, and the cytoplasmic fraction was obtained by centri-

Fig. 1. Specific reactivity of sera with the *M. penetrans* Triton X-114-extracted polypeptides, as shown by Western blotting. The pre-immune (lanes 1 and 3) and hyperimmune (lanes 2 and 4) sera from rabbit 1 and 2 were tested at 1:500 dilution. The monospecific anti-P35 preimmune (lane 5) and hyperimmune (lane 6) sera from rabbit 3 were tested at 1:100 dilution. The murine anti-P35 mAb 7 (lane 7) was tested at 1:2000 dilution. *M. penetrans*-seronegative (lane 8) and -seropositive (lane 9) human sera were tested at 1:100 dilution.

fugation (7000 g, 1 h, 4 °C). A volume of cytoplasmic fraction containing about 22 mg protein was loaded onto a nickel affinity Sepharose column (HisTrap; Pharmacia) and the histidine-tagged recombinant protein was eluted by an imidazole gradient (0–500 mM) in 50 mM NaH₂PO₄, 300 mM NaCl, 10% (v/v) glycerol (pH 8.0). The fractions containing the recombinant P35 (rP35Δ0) and the truncated P35 (rP35Δ3) contained 2 mg and 1.5 mg protein, respectively. Imidazole was removed by overnight dialysis at 4 °C against 50 mM NaH₂PO₄, 300 mM NaCl, 10% (v/v) glycerol (pH 8.0). The rP35Δ0 and rP35Δ3 proteins were identified on the basis of their molecular masses and their immunoreactivity with the polyclonal hyperimmune sera from rabbit 2. They were tested for antigenicity with other sera by Western blotting as described previously (Grau *et al.*, 1995).

RESULTS

Specificity of the anti-*M. penetrans* antibodies

The specificity of the various sera for polypeptides in the *M. penetrans* Triton X-114 extract was evaluated (Fig. 1). Sera from the two immunized rabbits, 1 and 2, (lanes 2 and 4) and the *M. penetrans*-seropositive human serum (lane 9) reacted with the three major polypeptides in this Triton X-114 extract: P30, P35 and P38. As expected, the hyperimmune serum from rabbit 3, which was immunized with a C-terminal synthetic peptide from P35, reacted with the P35 polypeptide only (Fig. 1, lane 6). mAb 7 reacted with the P35 polypeptide only (Fig. 1, lane 7).

To evaluate the neutralizing ability of the anti-*M. penetrans* antibodies, mycoplasma cells were cultured in the presence of the different sera. Mycoplasma growth was measured as CCU and growth of the control culture without antiserum used as the reference. mAb 7 and sera from immunized rabbits inhibited *M. penetrans* growth

similarly (–2logCCU). Sera from infected humans also inhibited mycoplasma growth but to different degrees (–1 to –2logCCU). The serum from a macaque experimentally infected with *M. penetrans* and pre-immune sera did not inhibit mycoplasma growth.

Cellular localization of the P35 antigen

The cellular localization of the P35 antigen was investigated using immunogold electron microscopy. mAb 7 bound to the surface of mycoplasmas, with both the tip and the cell body being labelled (Fig. 2a). To test whether P35 was only found at the cell surface, ultra-thin sections of mycoplasma cells were probed with mAb 7; the labelling was found only on the external surface of the *M. penetrans* plasma membrane (Fig. 2b). This demonstrates that the P35 lipoprotein is a polypeptide exposed externally on the cell surface. Presumably it is anchored to the lipid bilayer through its N-terminal acyl moiety.

P35 linear B-epitopes recognized by immunized animal sera

The formula described by Jameson & Wolf (1988) was used to predict the antigenicity of the mature P35 (Fig. 3). The calculation of a CO₅₀ value (1.35) for the AI allowed prediction of the main epitopes. There were six: one at the N-terminus, two at the C-terminus and three other regions in the middle of the sequence (Fig. 3). To localize sequential B-epitopes in P35, a set of 81 dodecapeptides covering the whole P35 primary sequence was synthesized. The peptides corresponding to the six predicted antigenic regions were: 1, 2, 15–17, 37–39, 44, 45, 73–75 and 76–78.

Fig. 2. Immunogold labelling of the P35 lipoprotein at the surface of *M. penetrans* cells. Electron microscopic examination of whole *M. penetrans* cells (negative staining; a) or ultra-thin cryosections (b) after incubation with the murine anti-P35 mAb 7. The gold particles conjugated to the secondary antibodies were 10 nm (a) or 5 nm (b) in diameter. Bars, 200 nm.

Fig. 3. Theoretical predictions of the antigenicity of P35. The dashed line indicates the calculated cut-off value. The black bars above the peaks indicate the peptide numbers of the synthetic peptides overlapping the P35 regions with the highest predicted antigenicity.

The reactivity of the 81 peptides with the various sera was tested by ELISA. Sera from pre-immune animals did not react with the peptides (Fig. 4a, d for rabbit 1 and macaque 9063, respectively). The sera from rabbits immunized with the Triton X-114 extract reacted with several peptides mostly clustered at the N- and C-termini of P35 (Fig. 4b, c for rabbits 1 and 2, respectively). In particular, successive pairs of peptides within the N-terminal end of P35 reacted with the serum from rabbit 1. It is likely that their antigenic determinants correspond to the common sequences: T¹⁷DGNTQQT²⁴ for peptides 4 and 5, T³⁷GALSKIY⁴⁴ for peptides 9 and 10, and A⁶⁴NPENYFT⁷¹ for peptides

16 and 17 (Fig. 4b). Similarly, pairs of peptides with common sequences were found to react with the serum from rabbit 2, T³⁷GALSKIY⁴⁴ for peptides 9 and 10, A⁶⁴NPENYFT⁷¹ for peptides 16 and 17, and N¹²⁹DLQTVLG¹³⁶ for peptides 32 and 33. The single peptide 14 (E⁵²TTSQIVKDIK⁶³) also reacted with this serum. Within the C-terminal region, clusters of peptides reacted: peptides 60–65 and 73–76 for rabbit 1, with two major reacting sequences corresponding to peptides 63 (N²⁴⁹AEKVLEKLG²⁶⁰) and 74 (D²⁹³STNNNKYT-VTL³⁰⁴), and peptides 73–77 for rabbit 2 with a major reacting sequence corresponding to peptides 76 and 77 (K³⁰⁵ATPNDGY³¹²). Both rabbit sera reacted with the

Fig. 4. Linear B-epitope mapping of *M. penetrans* P35 with sera from immunized animals. Sera from preimmunized rabbit 1 (a) and hyperimmune rabbits 1 and 2 (b, c) were tested at a 1:500 dilution. Preimmunized (d) and immunized (e) macaque 9063 sera were tested using a 1:200 dilution. All reaction peaks were tested for specificity by pre-incubation of the serum with the peptide prior to ELISA and the number of the corresponding peptide(s) is indicated only if there was a specific reaction.

C-terminal peptide 81 ($A^{321}KEEISFVATFS^{332}$). The reactivity of the serum from the immunized macaque 9063 was weaker. Only the 2 peptides, 8 ($A^{29}IKS-EVSLTGAL^{40}$) and 71 ($Y^{281}NCKFEAVKSEK^{292}$), were significantly recognized (Fig. 4e). The specificity of all these reactions was evaluated by inhibition assays in which the sera were pre-incubated with the corresponding peptide prior to their addition to the coated peptide in the ELISA. This pre-incubation resulted in the complete inhibition of the signal, confirming the specificity of the reaction (data not shown).

P35 linear B-epitope mapping using sera from *M. penetrans*-infected humans and macaques

Various reactivities were obtained with human sera (Fig. 5, Table 1). No peptide reacted with the sera from HIV- and *M. penetrans*-seronegative subjects and the background level was low (A_{490} around 0.03; Fig. 5a). In contrast, all sera from HIV-infected persons exhibited a higher background level with A_{490} close to 0.1 (Fig. 5b, c and d). Some of the 14 sera from HIV-seropositive but *M. penetrans*-seronegative patients reacted with a few peptides (Fig. 5b); only peptides 2, 4, 7, 12, 13 and 73 were significantly ($\geq 25\%$) recognized. Profiles obtained with sera from patients seropositive for both HIV and

M. penetrans were of two types: some exhibited low reactivity with the peptides (Fig. 5c), suggesting that they were directed against nonsequential rather than linear epitopes, whereas others recognized various peptides more strongly (Fig. 5d). Peptides 1, 2, 7, 11, 12, 13, 25, 37, 49, 60, 73, 74, 75, 76, 77 and 78 were significantly recognized ($\geq 25\%$) by at least seven of the 26 sera from this population. Most of these peptides were also recognized by the only HIV-seronegative and *M. penetrans*-seropositive serum that was tested (Table 1).

Many of the peptides recognized by *M. penetrans* seropositive sera were often clustered. The reaction profile shown on Fig. 5 (d) indicates that the serum recognized peptides 1, 12–14, 24, 25, 35–37, 48, 49, 60, 61 and others in the C-terminal region of the protein. Presumably this serum contains antibodies directed against the sequence common to clustered peptides: for example ($F^{97}TGEAYSV^{104}$) for peptides 24 and 25, which is not present on peptides 23 and 26. Similarly, for peptides 35–37 and 48–50, the epitopes probably correspond to the regions included in sequences $C^{145}DLIPNLKLNNG^{156}$ and $L^{197}NLKIDGLKISV^{208}$ respectively, which, interestingly, share the same NLK tripeptide.

Fig. 5. Linear B-epitope mapping of *M. penetrans* P35 with sera from *M. penetrans*-seropositive and -seronegative patients. The profile for one HIV- and *M. penetrans*-seronegative patient is shown in (a) and for one HIV-seropositive and *M. penetrans*-seronegative patient in (b). The profiles for two HIV- and *M. penetrans*-seropositive patient are shown in (c) and (d). All sera were tested at a 1:100 dilution. The calculated CO₅₀ values were 0.031, 0.129, 0.092 and 0.109, respectively.

The serum from the *M. penetrans*-infected macaque 91810 reacted specifically with peptides 7, 8, 14, 16, 17, 24, 25, 31, 61 and 64. Serum from the infected macaque 92926 reacted specifically with peptide 7, and that from infected macaque 8670 with peptides 25, 36, 38, 50 and 61. These results are summarized in Table 1. Note that peptide 25, which is the most widely recognized by human sera, was also recognized by two of the three experimentally infected macaques.

Involvement of the N-terminal acylated cysteine and of other post-translational modifications in the antigenicity of the *M. penetrans* Triton X-114-extracted antigens

The involvement of the N-terminal acylated structure of P35 in its antigenicity was evaluated by ELISA (Fig. 6a). Proteinase K treatment of the Triton X-114 extracted antigens totally abolished reactivity with nine *M. penetrans* seropositive sera. However, in one case (serum 3) the signal was not completely abolished after proteinase K treatment ($P=0.003$), suggesting that this serum reacted with short lipopeptides in the Triton X-114 extract. To evaluate the potential acylated C¹-binding ability of serum 3, the synthetic lipopeptide Pam₃-CYS, which mimics a classical lipoprotein N-

terminus, was used as the antigen in ELISA. Serum 3, but no other serum, reacted with Pam₃-CYS ($P=0.004$). Serum 3 binding to *M. penetrans* Triton X-114 antigens was significantly reduced (-18% , $P=0.01$) if it was preincubated with Pam₃-CYS prior to ELISA (data not shown).

Other putative post-translational modification sites in the deduced P35 amino acid sequence were predicted. In particular, there is a potential tyrosine-phosphorylation site, K²⁵²VLEKLG^{Y259}. P35 phosphorylation was evaluated by culturing *M. penetrans* in H₃³²PO₄-containing SP-4 followed by Triton X-114 fractionation, SDS-PAGE and autoradiography. Although a few *M. penetrans* polypeptides which sequestered in the aqueous phase after Triton X-114 extraction were phosphorylated, neither P35 nor any of the other polypeptides in the detergent phase were phosphorylated (data not shown). In addition, there was the possibility of an intramolecular C¹⁴⁵-C²⁸³ disulphide bond in P35. However, cysteine reduction by DTT and -SH alkylation with iodoacetamide did not alter the antigenicity of P35 as assessed by ELISA. This finding is consistent with the fact that the electrophoretic mobilities of reduced and non-reduced forms of the *M. penetrans* Triton X-114 antigens were indistinguishable in a polyacrylamide gel (data not shown).

Table 1. Amino acid sequence of the synthetic peptides and recognition frequencies by human or macaque sera

Peptide no.	Peptide sequence	Epitope prediction*	HIV ⁺ / <i>M. penetrans</i> ⁻ sera (n = 14)†	HIV ⁺ / <i>M. penetrans</i> ⁺ sera (n = 26)†‡	HIV ⁻ / <i>M. penetrans</i> ⁺ serum (n = 1)§	<i>M. penetrans</i> infected macaques (n = 3)
1	C ¹ SSTSENNNGNGN ¹²	+	2	12 ^b	+	
2	S ⁵ ENNGNGNGNGG ¹⁶	+	4	8		
7	E ²⁵ VTPAIKSEVSL ³⁶		4	8		++
8	A ²⁹ IKSEVSLTGAL ⁴⁰		2	4		+
11	S ⁴¹ KIYDTKTGTD ⁵²		2	8	+	
12	D ⁴⁵ TKTGTDR ⁵⁶		4	8	+	
13	G ⁴⁹ TDRETTSQMIV ⁶⁰		4	8		
14	E ⁵³ TTSQMIVKDIK ⁶⁴		1	2		+
16	K ⁶¹ DIKANPENYFT ⁷²	+	1	5		+
17	A ⁶⁵ NPENYFTNGEA ⁷⁶	+	0	5		+
24	T ⁹³ ESTFTGEAYSV ¹⁰⁴		0	3		+
25	F ⁹⁷ TGEAYSVWSAK ¹⁰⁸		2	17 ^c	+	++
31	K ¹²¹ QMDIKSINDLQ ¹³²		0	4		+
37	C ¹⁴⁵ DLIPNLKLNN ¹⁵⁶	+	2	9	+	+
38	P ¹⁴⁹ NLKLNNGT ¹⁶⁰	+	1	6		+
49	P ¹⁹³ VSDLNLKIDGL ²⁰⁴		0	7 ^b	+	+
50	L ¹⁹⁷ NLKIDGLKISV ²⁰⁸		2	5		+
60	P ²³⁷ AAVTLAEDRT ²⁴⁸		0	7 ^b	+	+
61	T ²⁴¹ LAEADRTNAEK ²⁵²		3	6		+
63	N ²⁴⁹ AEKVLEKLG ²⁶⁰		1	2		+
73	K ²⁸⁹ SEKDSTNNNKY ³⁰⁰	+	4	15 ^a	+	
74	D ²⁹³ STNNNKYTVTL ³⁰⁴	+	3	8		
75	N ²⁹⁷ NKYTVTLKATP ³⁰⁸	+	2	9		
76	T ³⁰¹ VTLKATPNDGY ³¹²	+	2	7		
77	K ³⁰⁵ ATPNDGYFWED ³¹⁶	+	2	11 ^a	+	
78	N ³⁰⁹ DGYFWEDGTNG ³²⁰	+	3	9		

* Epitopes predicted according to Jameson & Wolf (1988). The peptides predicted to be the most antigenic are indicated by +. See also Fig. 3.

† Number of sera with $A_{492} > CO_{50}$.

‡ Significant differences between HIV⁺ and *M. penetrans*⁻ versus *M. penetrans*⁺ populations are indicated by italic superscript characters: a, $P < 0.08$; b, $P < 0.05$; c, $P < 0.001$.

§ The peptides for which the A_{492} value was higher than the determined CO_{50} (0.085) are indicated as +.

|| The number of + symbols indicates the number of animal sera, out of 3, reacting with the peptide.

There are two putative N-glycosylation sites, N¹⁵⁵GT¹⁵⁷ and N¹⁷⁶VT¹⁷⁸, and one putative O-glycosylation site (Hansen *et al.*, 1995), T²⁷, in the deduced sequence of P35. We previously demonstrated by periodic acid-Schiff staining after SDS-PAGE of *M. penetrans* Triton X-114 extracted polypeptides that P35 is not glycosylated, at least within the detection limits of this method (Neyrolles *et al.*, 1998). We also demonstrated that no *M. penetrans* polypeptide contains either glucose or mannose (data not shown) by Western blotting using ConA as a probe.

Nonsequential B-epitopes of the P35

To obtain a recombinant P35 we changed the two in-frame TGA codons in the *p35* gene to TGG to allow efficient expression in *E. coli*. To facilitate the puri-

fication of the recombinant protein rP35Δ0, the *p35* gene was inserted into the vector pQE31 giving pQEP35Δ0, which resulted in the addition of an His₆ N-terminal tag. By directed mutagenesis of pQEP35Δ0, the recombinant product rP35Δ3 (21.6 kDa) which corresponds to the first 182 amino acids of P35 was also produced (Fig. 7a). Culture extracts were subjected to Ni-affinity chromatography and the fractions analysed by SDS-PAGE. Protein rP35Δ0 was recovered from the *E. coli* cytoplasmic fraction with only two minor polypeptides using this one-step purification procedure (Fig. 7b, lanes 1 and 2). In contrast, using similar conditions of elution from the affinity column, the purity of rP35Δ3 preparations was always lower (Fig. 7b, lanes 3 and 4).

The antigenicity of both proteins was studied by Western blotting using a panel of sera (Fig. 7c). The rabbit polyclonal serum, used as a positive control,

Fig. 6. Involvement of the P35 polypeptide and acylated N-terminal moiety in its antigenicity. (a) Ability of human *M. penetrans*-seronegative (1) vs -seropositive (2–10) sera, used at 1:100 dilution, to react with proteinase K-treated *M. penetrans* Triton X-114 extracted antigens, as determined by ELISA. *M. penetrans* Triton X-114 extract digested (hatched) or undigested (black) with proteinase K was used as the antigen. (b) Pam₃-CYS lipopeptide used as the antigen.

reacted with both rP35Δ0 and rP35Δ3 (Fig. 7c; lane 10), whereas the corresponding pre-immune serum was negative (Fig. 7c; lane 9). Six human sera, selected for their reactivity with the native P35 from the *M. penetrans* Triton X-114 extract were tested. All reacted with rP35Δ0 but only one reacted with rP35Δ3 (Fig. 7c; lane 6).

DISCUSSION

The P35 sequence contains a typical lipoprotein signal sequence, consistent with previous results showing that P35 is acylated (Ferris *et al.*, 1995). Mycoplasma lipoproteins are thought to be exposed on the cell

surface and anchored to the lipid bilayer through their N-terminal lipid moiety (Wieslander *et al.*, 1992). We used a monoclonal anti-P35 antibody to demonstrate that P35 is located on the external surface of the *M. penetrans* plasma membrane. It has been suggested that P35 is involved in *M. penetrans* cytoadherence (Giron *et al.*, 1996). However, our results indicate that P35 is distributed all over the plasma membrane and is not confined to the tip structure, which mediates cytoadherence (Lo *et al.*, 1992). This is in contrast with other mycoplasmal adhesins and adhesin-related polypeptides such as P1, P30, P90 and HMW3 in *M. pneumoniae* or MgPa in *M. genitalium*, which have been shown to be clustered at the cell tip (for a review see Krause, 1996). However, the P50 adhesin of *M. hominis*, a mycoplasma that lacks the tip structure, was shown to be distributed all over the cell surface (Henrich *et al.*, 1993). Therefore, our findings do not exclude that P35 plays a role in *M. penetrans* cytoadherence. The abundance of P35 and its exposure at the cell surface are in agreement with this lipoprotein being targeted by the humoral arm of the host immune system (Grau *et al.*, 1995; Wang *et al.*, 1992). To identify the major linear antigenic regions of P35, we used a set of synthetic peptides covering the entire P35 amino acid sequence. This ELISA-based technique is now widely used to define sequential epitopes in a protein and its advantages and disadvantages have been reviewed by Geysen *et al.* (1987). A similar approach has previously been used for T-epitope mapping in various bacterial antigens including the *Mycobacterium bovis* P38 (Pollock *et al.*, 1995), and for B-epitope mapping of the Pg-II fimbria from *Porphyromonas gingivalis* (Ogawa *et al.*, 1995) and of the MB antigen from *U. urealyticum* (Zheng *et al.*, 1996). This strategy is particularly suitable for mapping sequential epitopes of three to eight amino acid residues (Arnon & Van Regenmortel, 1992; Geysen *et al.*, 1987). To map epitopes as small as four amino acid residues, we used dodecapeptides, each one overlapping the previous by eight amino acid residues. It is unlikely that the changes in signal in our epitope characterization experiments were due to different amounts of the peptides bound to the microtitre wells. The results obtained with immunized animal sera (rabbit and macaque) are partly in accordance with the predicted antigenic profile of P35 (Fig. 3); peptides predicted to be highly antigenic were well recognized by hyperimmune rabbit sera (e.g. T¹⁷DGNTQQT²⁴ with a mean AI of 1.081). However, other amino acid sequences, such as T³⁷GALSKIY⁴⁴ and A³²¹KEEISFVATFS³³², which were predicted to be poorly antigenic (mean AI of -0.206 and -0.012, respectively) were also well recognized by these sera. Similarly, peptide 14 (E⁵³TTSQLIVKDIK⁶⁴), which was predicted to be weakly antigenic (mean AI of 0.250), gave the strongest reaction with hyperimmune serum from rabbit 2. Peptides encompassing amino acid residues N¹⁵⁴NGT¹⁵⁷ were predicted to be highly antigenic (AI 1.700), but were not recognized by hyperimmune sera. Finally, the immunized macaque 9063 serum reacted only, but very significantly, with peptides A²⁹IKSEVSLTGAL⁴⁰ (predicted to be poorly antigenic)

Fig. 7. Purification and antigenicity of the two recombinant P35 proteins. (a) Schematic representation of the recombinant proteins rP35Δ0 and rP35Δ3 obtained by directed mutagenesis. (b) SDS-PAGE analysis of fractions containing rP35Δ0 and rP35Δ3. rP35Δ0 and rP35Δ3 production was induced from *E. coli* transformants containing the plasmids pQEP35Δ0 and pQEP35Δ3, respectively. The polypeptide content of cytoplasmic fractions from these transformants was analysed (*E. coli* with pQEP35Δ0, lane 1; *E. coli* with pQEP35Δ3, lane 3). Fractions eluted from the Ni-affinity chromatography containing rP35Δ0 and rP35Δ3 are shown in lanes 2 and 4, respectively. (c) Evaluation of antigenicity of rP35Δ0 and rP35Δ3 by Western blotting. The pre-immune (lane 9) and hyperimmune (lanes 10) rabbit 2 sera were tested at a 1:500 dilution. Sera from *M. penetrans*-seropositive (lanes 1–6) and -seronegative (lanes 7, 8) humans were tested at a 1:50 dilution. The development of the enzymic reaction was stopped simultaneously for all Western blot strips tested with human sera.

and Y²⁸¹NCKFEAVKSEK²⁹² (mean AI of -0.237 and 0.512, respectively).

The reaction profiles for infected macaques and patients differ significantly from those for immunized animals. This is most likely due to the combination of at least two factors: the P35 polypeptide used to inoculate rabbits was partially denatured and the repertoire of B epitopes recognized in different animal species is known to vary (Berzofsky, 1985).

The profiles obtained with infected macaques and patients shared several common features. In particular, peptide 25 was recognized by two infected macaques and by about 66% of the *M. penetrans* seropositive patients ($P < 0.001$). The HIV serological status of the

human donors clearly interfered with this epitope-mapping analysis by generating a high level of background. This difficulty was probably due to the polyclonal B cell activation and IgG hypersecretion which occur during HIV infection (Lane *et al.*, 1983). However, it was not possible to limit our study to HIV-seronegative patients due to the low incidence of *M. penetrans*-seropositivity in this population (0.3–1.3%; Brenner *et al.*, 1996). The heterogeneity of the antibody response observed in HIV-infected patients was more likely due to a combination of factors which include the diversity of their MHC genes and of their Ig gene repertoire, and the effects of HIV infection on the repertoire of immune cells. MHC genes act indirectly, by influencing the level and specificity of helper T

lymphocytes, which are required for activation of B cells to make antibodies against protein antigens. Examples of the MHC control of the specificity of antibody production include responses to the hepatitis B surface antigen (Milich *et al.*, 1984) and to peptides of myelin basic protein (Fritz *et al.*, 1985). The perturbation of the repertoire of T helper cells is one of the landmarks of HIV infection and has indirect effects on antibody production (Gorochov *et al.*, 1998).

In spite of this heterogeneity in the antibody response, peptides 1, 2, 37, 38 and 73–78, which were predicted to contain the most antigenic amino acid residues (Fig. 3), were significantly ($\geq 25\%$) recognized by sera from HIV- and *M. penetrans*-seropositive patients. Two peptides reacted with most *M. penetrans*-seropositive sera, peptides 1 (46.1%) and 25 (65.4%), but only with 14% of *M. penetrans*-seronegative sera ($P < 0.05$ and 0.001 , respectively). The recombinant form of P35, rP35 Δ 0, was recognized by all the sera from *M. penetrans*-seropositive patients tested in this study. Only one in six of these human sera reacted with rP35 Δ 3, indicating that either the region corresponding to the P35 C-terminal moiety contains major linear epitopes or that conformational determinants involving this region are recognized by patient sera. These two possibilities are not exclusive and were supported by our data from ELISA with the set of synthetic peptides. Indeed, among the human sera which reacted with P35 on Western blots, some did not show any significant reactivity with the set of P35-derived peptides, whereas others which were found to contain antibodies to linear B-epitopes were reactive with numerous peptides from the P35 C-terminus. In addition, the rabbit PAb 2, which reacted with rP35 Δ 3, recognized linear B-epitopes located at the N-terminus of P35.

The linear epitopes defined using the panel of 81 overlapping peptides only represent antigenic regions with no post-translational modifications. Therefore, we also investigated the possibility that other features of *M. penetrans* P35 could contribute to its antigenicity, in particular its N-terminal lipid moiety. Many lipid moieties of bacterial lipoproteins are strongly immunogenic, and are potent activators of B cells and macrophages. These properties were documented by the use of structural analogues of the N-terminal lipopeptide domain of Braun's lipoprotein from *E. coli* (Hauschildt *et al.*, 1990; Melchers *et al.*, 1975), and with lipidated or non-lipidated forms of the *B. burgdorferi* OspA (Erdile *et al.*, 1993). It was also shown that the N-terminal lipid moiety of the P47 major membrane antigen from *T. pallidum* is not an antigenic structure (Weigel *et al.*, 1992). Similarly, Shouls *et al.* (1991) demonstrated that the lipid moiety of the TmpA and TmpC surface lipoproteins from *T. pallidum* strongly enhanced the antibody response in animals, acting as both carrier and adjuvant, but that all the anti-TmpA and -TmpC antibodies were exclusively directed against the polypeptide parts of these lipoproteins. We showed that the protein moiety of the *M. penetrans* Triton X-114 antigens carried the majority of the B-epitopes recog-

nized by human sera. Indeed, proteinase K treatment of these antigens abolished their reactivity. However, for one of 9 sera (serum 3, Fig. 6a), the proteinase K treatment did not entirely eliminate the immune recognition of *M. penetrans* Triton X-114 antigens. This serum bound a synthetic lipopeptide (Fig. 6b) and this binding was, at least in part, competitive with binding to *M. penetrans* Triton X-114 antigens. To our knowledge, this is the first data indicating that, at least in a few cases, human sera can react with the N-terminal lipid moiety of bacterial lipoproteins.

We have evaluated whether other post-translational modifications of P35 were involved in its antigenicity. The putative intramolecular C¹⁴⁵–C²⁸³ disulphide bond did not contribute to P35 antigenicity, whereas such a disulphide bond was found to be essential for the antigenicity of the P29 polypeptide from *M. fermentans* (Theiss *et al.*, 1996). Although it was suggested by Andreev *et al.* (1995) that P35 might be phosphorylated, our data indicated that it was not the case. Finally, our preliminary data did not suggest that P35 is glycosylated, but this modification cannot yet be excluded.

We are currently investigating the possibility of using a cocktail of P35-derived synthetic peptides (such as both peptides 1 and 25) or adding this cocktail to Triton X-114 antigens, to improve the *M. penetrans* serological assay. In addition, we are addressing whether rP35 Δ 0 can be used as the antigen, instead of the crude Triton X-114 extract, in a new version of the *M. penetrans*-specific ELISA.

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