Lipid-restricted recognition of mycobacterial lipoglycans by human pulmonary surfactant protein A: a surface-plasmon-resonance study

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The human pulmonary surfactant protein A (hSP-A), a member of the mammalian collectin family, is thought to play a key defensive role against airborne invading pulmonary pathogens, among which is *Mycobacterium tuberculosis*, the aetiological agent of tuberculosis. hSP-A has been shown to promote the uptake and the phagocytosis of pathogenic bacilli through the recognition and the binding of carbohydrate motifs on the invading pathogen surface. Recently we identified lipomannan and mannosylated lipoarabinomannan (ManLAM), two major mycobacterial cell-wall lipoglycans, as potential ligands for binding of hSP-A. We demonstrated that both the terminal mannose residues and the fatty acids are critical for binding, whereas the inner arabinosyl and mannosyl domains do not participate. In the present study we developed a surface-plasmon-resonance assay to analyse the molecular basis for the recognition of

ManLAM by hSP-A and to try to define further the role of the lipidic aglycone moiety. Binding of ManLAM to immobilized hSP-A was consistent with the simplest one-to-one interaction model involving a single class of carbohydrate-binding site. This observation strongly suggests that the lipid moiety of ManLAM does not directly interact with hSP-A, but is rather responsible for the macromolecular organization of the lipoglycan, which may be necessary for efficient recognition of the terminal mannosyl epitopes. The indirect, structural role of the lipoglycan lipidic component is further supported by the complete lack of interaction with hSP-A in the presence of a low concentration of mild detergent.

Key words: lectin, lipoarabinomannan, tuberculosis.

INTRODUCTION

Mammalian collectins are essential components of the innate immune system, involved in the clearance of a wide range of invading pathogens [1–3]. When considering airborne infections, the quantitatively major collectin of the lung alveoli surfactant, namely pulmonary surfactant protein A (SP-A), is of special interest, since it may provide the first line of host defence against specific pulmonary pathogens [4]. SP-A has been shown to promote the uptake and the phagocytosis of *Mycobacterium tuberculosis*, the causal agent of tuberculosis [5,6] and of *Mycobacterium bovis* BCG (BCG), the vaccinating strain [7].

Like all other collectins, human SP-A (hSP-A) results from a complex oligomeric association of trimeric subunits. In this trimeric subunit, each 25–35 kDa monomer consists of four distinct domains: a cysteine-rich N-terminal domain followed by a collagen domain linked to a C-terminal carbohydrate-recognition domain (CRD) through a short hydrophobic zone of approximately about 45 residues termed the ‘neck’ region (for a review, see [8]). The main physiological form of hSP-A found in the lung surfactant is an octadecameric complex, formed by the assembly of six trimers, adopting a characteristic three-dimensional structure similar to that for the serum mannose-binding protein (MBP) and often compared with a ‘bunch of tulips’ [1]. hSP-A has been shown to bind specifically to various micro-organisms by interacting with microbial surface carbohydrates via its C-terminal domain. Various biochemical and

molecular studies have shown that this lectin activity is Ca²⁺-dependent [9]. Moreover, it has been shown that the hSP-A binds specifically to monosaccharides having *trans* di-equatorial hydroxy groups (in M relative helical arrangement [10]), such as mannose or glucose. This sugar specificity and the high similarity of the primary sequence of hSP-A lectin domain to that of MBP (≈ 40% identity, including the consensus motifs characteristic of the mannose-binding C-type lectins), support a similar carbohydrate recognition and binding scheme for MBP and SP-A [11].

However, although the monosaccharide-recognition mechanism and the specificity of these lectins have been well documented, little is known concerning the putative microbial ligands of hSP-A. Several studies provided evidence that the binding of collectins to Gram-negative bacteria is mediated by the external lipopolysaccharides [12,13] or outer-membrane proteins [14], while interactions with fungi occur mainly through their envelope glycoproteins [15]. Regarding mycobacteria, Weikert et al. [16] suggested the presence of a galactosyl-containing ligand in *M. bovis* BCG on the basis of monosaccharide inhibition experiments. On the other hand, Pasula et al. [17] proposed a mannose-rich antigenic glycoprotein (≈ 60 kDa) in *M. tuberculosis*. Recently, in order to identify the putative mycobacterial ligands of hSP-A, we tested several purified glycoconjugates of the *M. bovis* BCG envelope for their ability to bind to purified hSP-A. Among these compounds we found that only the two major mycobacterial envelope lipoglycans, namely the mannosylated

Abbreviations used: AG, mycobacterial arabinogalactan; BALF, bronchoalveolar lavage fluid; GL, mycobacterial glucan; hSP-A and -D, human surfactant proteins A and D; LM, lipomannan; ManLAM, mannose-capped lipoarabinomannan; ManAM, mannose-capped arabinomannan; MBP, mannose-binding protein; BCG, *Mycobacterium bovis* BCG (bacille Calmette–Guérin); ScMan, *Saccharomyces cerevisiae* mannan; SPR, surface plasmon resonance; CRD, carbohydrate-recognition domain; PAMPs, pathogen-associated molecular patterns; RU, resonance unit; PiLAM, phosphoinositol-capped lipoarabinomannan.

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Figure 1 Structures of the major mannosylated lipoglycans and polysaccharides isolated from cell wall of slow-growing mycobacterial strains such as *M. tuberculosis*

(a) LM; (b) ManLAM; (c) AM (note that $\rightarrow$ means $\rightarrow$).

lipoarabinomannan (ManLAM) and the lipomannan (LM) (Figure 1) are recognized by hSP-A. Thus we proposed that they constitute potential ligands for the attachment of hSP-A to the surface of BCG [10]. Surprisingly none of the mannose- or glucose-containing polysaccharides tested was able to interact with hSP-A. Further attempts to understand these unexpected results suggested that the hydrophobic component of the lipoglycans is necessary for the recognition and the binding of the mannosyl epitopes to the lectin domains of the hSP-A. The solid-phase presentation mode used in this previous study, however, prevented any definitive conclusion from being drawn regarding the role of the hydrophobic moiety. Indeed, we were unable to rule out the possibility that the absence of hSP-A interaction with the polysaccharides was due to a differential qualitative adsorption of the glycoconjugates, resulting from the absence of the hydrophobic aglycone moiety.

To overcome these limitations, we developed a new hSP-A binding micro-assay based on surface plasmon resonance (SPR). This assay permits the analysis of the interaction between the immobilized hSP-A and mycobacterial ligands in solution. In the present paper we report for the first time the coupling of an acidic high-molecular-mass octadecameric protein to a newly developed biosensor chip containing a shorter, carboxymethylated dextran chain. This system was then used to study the interaction of hSP-A with the complex mycobacterial ManLAM. This enabled us to confirm our previous observations and to provide insights into the role of the aglycone moiety in controlling the carbohydrate-lectin interaction. These findings may have important consequences in terms of understanding the mechanisms of antigen recognition by mammalian collectins.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals were obtained from Sigma (St. Louis, MO, U.S.A.). Arabinogalactan (AG) from *Mycobacterium smegmatis* MC²155 and glucan (GL) from *M. tuberculosis* H37Rv were generously provided by Dr V. Puech and Dr A. Lemassu at one of our institutions [Institut de Pharma-

cologie et Biologie Structurale (I.P.B.S.)]. Bronchoalveolar lavage fluids (BALF) from patients with alveolar proteinosis were generously given by Dr M. C. Prévost (INSERM U326, CHU Purpan, Toulouse, France). Trimannoside-BSA neo-glycoconjugate (Man₃-BSA) ($\approx$ 13 mol of trimannoside/mol of protein) was synthesized by Dr J. Prandi (IPBS) as previously described [18].

Methods

Preparation of mycobacterial cell wall glycoconjugates

LAMs, LM and mannosylated arabinomannan (ManAM) from *M. bovis* BCG 1173P2 were purified in our laboratory as described in [19–22]

Preparation of deacylated LAM (dManLAM)

M. bovis BCG ManLAM (200 μ g) was incubated (2 h) at 37 °C in 0.1 M NaOH (final volume 200 μ l). After neutralization with HCl (0.1 M), the reaction product was dialysed using 6–8000-Da-molecular-mass-cut-off membranes (Spectra/Por[®]; Spectrum Medical Industries, Houston, TX, U.S.A.) against deionized water [10].

Preparation of α -mannosidase-treated LAM (aManLAM)

M. bovis BCG ManLAM (200 μ g) was incubated (6 h) at 37 °C in 30 μ l of jack-bean (*Canavalia ensiformis*) exo- α -mannosidase solution (2 mg/ml in 100 mM sodium acetate/1 mM ZnSO₄, pH 4.5). After a second addition of 50 μ l of enzyme, the solution was incubated overnight and then dialysed against 50 mM NH₄CO₃, pH 7.6. Elimination of α -mannosidase was achieved by heat denaturation (2 min, 110 °C), followed by overnight tryptic digestion (37 °C, trypsin/ α -mannosidase ratio 1:50, w/w). After extensive dialysis against deionized water, the reaction products were analysed by capillary zone electrophoresis as previously described [10].

Purification of hSP-A

Human SP-A (hSP-A) was purified from bronchoalveolar lavage fluid from patients with alveolar proteinosis [23] with some modifications [10]. Briefly, alveolar surfactant was isolated from 150 ml of BALF by centrifugation (20000 g, 4 °C, overnight). The pellet was delipidated by extraction with 100 ml of butan-1-ol. After centrifugation (5000 g, 4 °C, 30 min), the pellet, containing the butanol-insoluble proteins, was dried under a gentle stream of nitrogen. The resulting powder was suspended in 50 ml of 5 mM Tris/HCl (pH 7.4)/100 mM NaCl/20 mM n-octyl glucoside to solubilize the serum proteins. Insoluble hSP-A was collected by ultracentrifugation (200000 g, 4 °C, 30 min). The pellet was washed twice in the same buffer and then suspended in 5 ml of 5 mM Tris/HCl, pH 7.4. The n-octyl glucoside was eliminated by extensive dialysis against the same buffer and the residual insoluble material was removed by centrifugation (100000 g, 4 °C, 30 min). The supernatant was adjusted to 100 mM NaCl/10 mM CaCl₂ and applied on a mannose-agarose column pre-equilibrated in 5 mM Tris/HCl (pH 7.4)/100 mM NaCl/10 mM CaCl₂ for further purification of functionally active SP-A. After washing the column by 3 bed vol. of the same buffer, hSP-A was eluted with 5 mM Tris/HCl (pH 7.4)/100 mM NaCl/5 mM EDTA and dialysed against 5 mM Tris/HCl, pH 7.4. The amount of purified hSP-A was estimated using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and the purity was checked by SDS/PAGE, revealed by

staining (Coomassie Blue) and by immunodetection using rabbit anti-SP-A antiserum [10].

SPR assays

The SPR binding assays were performed on a BIAcore 1000 instrument upgraded to 2000 specifications (BIAcore AB, Uppsala, Sweden). Pre-concentration studies were conducted on pioneer F1 sensor chips (BIAcore AB, Uppsala, Sweden) at a flow rate of 5 μ l/min. For each pH value tested (3.5, 4.5 and 5.5), 35 μ l of hSP-A, containing from 5 to 100 μ g/ml in 10 mM sodium acetate, was injected. A regeneration cycle (injections of 10 μ l of 10 mM HCl and 10 μ l of 10 mM NaOH) was performed between each injection in order to retrieve the baseline.

hSP-A was immobilized on the dextran matrix of a pioneer F1 sensor chip using the standard amine immobilization kit supplied by the manufacturer [24]. Briefly, following activation of the chip surface with 50 mM *N*-hydroxysuccinimide and 200 mM *N*-(dimethylaminopropyl)-*N'*-ethylcarbodi-imide, hSP-A (40 or 100 μ g/ml in 10 mM sodium acetate at pH 3.5, 4.5 or 5.5) was injected through the chip cell at a flow rate of 5 μ l/min. Subsequently, the remaining sites that had not reacted were inactivated by exposure to 1 M ethanolamine, pH 8.5.

Binding analysis was performed at a flow rate of 15 μ l/min in 5 mM Tris/HCl (pH 7.4)/100 mM NaCl/10 mM CaCl₂. Aliquots of the different glycoconjugates tested, at a concentration of 100 μ g/ml or increasing concentrations of ManLAM, were injected over 1.30 min. At the end of the injection, the complexes formed were allowed to dissociate for 6 min, then one cycle of regeneration (injection of 5 mM EDTA) was performed. Injections were separated by an equilibration delay of 15 min, with binding buffer at a flow rate of 1 μ l/min. Control tests were obtained by injection of the respective analyte solutions on a sham-treated chip (submitted to the activation-deactivation coupling protocol). Primary sensorgrams were analysed by non-linear curve fitting using the BIAevaluation 2.1 software (BIAcore AB).

RESULTS

Immobilization

Owing to the limiting physical and chemical properties of hSP-A (molecular mass 650 kDa; pI 4.0–4.5) [25], pre-concentration experiments, varying either the ligand concentration or the pH of the solution, were necessary to determine the optimum conditions

Figure 2 Electrostatic adsorption of hSP-A to non-activated carboxymethyl-dextran sensor chips as a function of hSP-A concentration at different pH values

The points are derived from individual sensorgrams recorded with increasing hSP-A concentration in 10 mM sodium acetate solution adjusted to the corresponding pH.

Table 1 Immobilization of hSP-A on an activated carboxymethyl-dextran sensor chip under different conditions of pH and concentration

Conditions	Response (RU)	
	First injection	Second injection
F1 sensor chip		
pH 3.5, 40 μ g/ml hSP-A	550 (0.7 ng*)	—
pH 4.5, 100 μ g/ml hSP-A	1300 (1.5 ng)	2770 (3.3 ng)
pH 3.5, 100 μ g/ml hSP-A	3530 (4.2 ng)	3530 (4.2 ng)
CM5 sensor chip		
pH 3.5, 100 μ g/ml hSP-A	9800 (11.7 ng)	12740 (15.2 ng)

* Resonance unit response expressed as the quantity (ng) of protein fixed on a 1.2 mm² support surface [27].

for hSP-A immobilization. The plot of the SPR response versus the concentration at different pH values (Figure 2) indicated that, at pH 5.5, there was almost no electrostatic interaction between hSP-A and the negatively charged carboxy groups of the dextran on the biosensor chip. This is consistent with the fact that, according to the pI of hSP-A, the protein is negatively charged at this pH. At pH 4.5, no or very little interaction was observed at concentrations below 50 μ g/ml. Above this value, the response signal rapidly reached a plateau of about 400 resonance units (RU), suggesting saturation of the dextran matrix surface. At pH 3.5, a similar plateau of 400 RU was observed from 10 to 50 μ g/ml. Above this concentration, the response increased in a concentration-dependent manner to attain a value of about 1200 RU at 100 μ g/ml. A value of 50 μ g/ml corresponds to the critical aggregate concentration of hSP-A [12]. Therefore the data suggest that, under the conditions used, hSP-A may experience a different conformation or aggregation state (as reported for porcine SP-A [26]) leading to the appearance of positive charges favouring electrostatic attraction. We verified, by using a solid-phase binding assay, that the modifications induced by such changes in concentration and pH are reversible and therefore do not alter the functionality of the carbohydrate-binding domains when the pH is shifted back to neutral (results not shown).

On the basis of the pre-concentration experiments, we attempted to immobilize hSP-A on the newly developed F1 sensor chips (Table 1). As suspected, no immobilization was detected at pH 5.5 whatever the concentration used, or at pH 4.5 at concentrations below 50 μ g/ml. At this pH we were able to obtain a low level of immobilization by raising the concentration to 100 μ g/ml (Table 1). The maximum level of immobilization, on the F1 sensor chips, was achieved at pH 3.5 and with 100 μ g/ml of protein (Table 1). Under these conditions, the support was rapidly saturated ($\approx$ 120 s) and a second injection is totally ineffective (Figure 3). We obtained a maximum SPR response of 3500 RU corresponding to about 4.2 ng of immobilized protein, assuming that the mass/response relationship (1000 RU $\approx$ 1 ng/mm² [27]) can be applied on the F1 support. In comparison, about five-fold more protein could be immobilized on standard CM5 sensor chips under the same conditions (Figure 3 and Table 1). This difference in the amount of protein immobilized on these two supports can be correlated to the 60% reduction of the dextran matrix chain length of the F1 support (40 nm) compared with the standard CM5 chip (100 nm). Nevertheless, although higher levels of immobilization may result in a higher maximum analyte binding capacity, preliminary binding studies using hSP-A grafted on to CM5 sensor chips gave much

Figure 3 Sensorgrams of hSP-A immobilization via amine coupling chemistry to short-chain carboxymethyl-dextran F1 (continuous line) and standard CM5 (broken line) supports

The immobilization reaction was run at constant flow rate (5 $\mu\text{l}/\text{min}$), and the different steps were: (1) activation of the support carboxy groups by *N*-hydroxysuccinimide/*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodi-imide; (2) washing (10 mM sodium acetate, pH 3.5); (3) hSP-A (100 $\mu\text{g}/\text{ml}^{-1}$), first injection; (4): washing; (5) hSP-A, second injection; (6) washing; (7) de-activation of the remaining reactive groups by 1 M ethanolamine.

lower-intensity responses (results not shown). An explanation for this apparent discrepancy is that the excess of hSP-A bound to the CM5 is probably less accessible to the analyte, because it is buried in the depths of the dextran layer. In addition, by diminishing the depth of the hSP-A-bound dextran layer, the system tends to an ideal two-dimensional system, minimizing the dramatic limitations caused by the rate of the analyte mass transport through the matrix (as observed in previous experiments using CM5 sensor chips; S. Sidobre and M. Rivière, unpublished work).

Since SP-A is known to reversibly self-aggregate under the conditions we used for its immobilization [12,26], we feared that the highest level of binding observed at pH 3.5 resulted from the immobilization of high-mass aggregates of non-covalently associated molecules. To rule out this possibility, the sensor chips were submitted to multiple regeneration cycles using acidic (10 mM HCl), alkaline (10 mM NaOH) or neutral (5 mM EDTA, pH 7.4) conditions. Only a limited response decrease (< 10%) was observed under acidic or neutral conditions at the end of ten cycles (results not shown). By contrast, the use of alkaline buffer led to a significant signal reduction after each regeneration step, suggesting a loss of material from the surface. This could be attributed to the alkaline lability of the disulphide bonds involved in the protein oligomerization. Thus these results strongly support the fact that the maximal response observed at pH 3.5 resulted from the immobilization by covalent linkage of non-aggregated hSP-A molecules on the sensor chip surface.

Mycobacterial lipoglycan binding to immobilized hSP-A

Attempts to verify the integrity of the carbohydrate binding functions of the immobilized protein using commercially available ligands such as the mannosylated-BSA (Man-BSA) or *Saccharomyces cerevisiae* mannan (ScMan) were unsuccessful when tested at 100 $\mu\text{g}/\text{ml}$ (results not shown). These observations are consistent, however, with our previous report indicating a very limited interaction between hSP-A and solid-phase-presented ScMan [10]. Thus, to verify that immobilization does not affect/alter the collectin properties, we tested the ability of hSP-A to interact with several mannose- or glucose-containing purified mycobacterial cell-wall components (Figure 4). Qualitative analysis of the sensorgrams revealed striking differences between the behaviour of polysaccharides and lipoglycans. Indeed, the arabinomannan [20] (Figure 4, bottom panel), the

Figure 4 Interaction of the major mycobacterial cell-wall polysaccharides and lipoglycans (100 $\mu\text{g}/\text{ml}$) with immobilized hSP-A (top panel) and schematic structures of the ligands used in this assay (bottom panel)

In the bottom panel, mycobacterial glucan (Glu) has a glycogen-like structure composed of repeating units of five or six 4-*O*-linked α -D-glucose residues substituted at position 6 with mono- or di-glucosyl residues [29]. The arabinogalactan (AG) released by alkaline hydrolysis of the mycobacterial cell-wall skeleton is composed of a linear homogalactofuranose core substituted by three arabinan chains of approx. 27 arabinofuranose residues each [28].

arabinogalactan [28] and the glucan [29] gave only a limited response. This supports the idea that polysaccharides interact very weakly with hSP-A. By contrast, the LM and the ManLAM bound strongly to the immobilized protein. ManLAM seemed to give a stronger response than LM (Figure 4), which is not what we found in the solid-phase binding assays. This obvious difference in response intensity can be attributed to the SPR detection principle and results from the difference between the average molecular masses of these two analytes (molecular mass of ManLAM $\approx$ 16000 Da [30]; molecular mass of LM $\approx$ 7500 Da; G. Puzo, unpublished work). Indeed, considering that the intensity of the response is proportional to the analyte mass and that it complies with the mass/response relationship

Figure 5 Corrected relative binding of mycobacterial ligands to immobilized hSP-A

The direct relative response derived from the sensorgram maximum values, measured at the end of the association (injection) period, was weighted by the molecular-mass ratio of the ligands. The average molecular-mass values used in the calculations were as follows: ManLAM, 17.4 kDa [30]; LM, 7.5 kDa (G. Puzo, unpublished work); ManAM, 16 kDa [20]; Glu, 120 kDa [29]; and AG, 15 kDa [28].

Table 2 Identification of the lipoglycan structural motifs required for the interaction (responses are expressed relative to that of ManLAM)

Abbreviations: LGly, lipoglycan; Gly, glycan; n.d., not detected. Structures of the compounds, but not that of ScMan, are shown in (b). The structure of LM, not listed in (a), is also shown. The underlining differentiates the polymeric carrier moiety (glycan, lipoglycan, protein) from the chemically defined saccharidic motifs susceptible to interact with a lectin receptor.

	Non-reducing end terminal residue	Relative response (%)
(a) Compound		
ManLAM <i>M. bovis</i> BCG	[Man α 1 $\rightarrow$ 2] _n Man α 1 $\rightarrow$ 5Araf $\rightarrow$ <u>LGly</u>	100
LAM <i>M. smegmatis</i>	<u>myo</u> -inositol 1-phosphate-5Araf $\rightarrow$ <u>LGly</u>	< 10
α -Mannosidase-treated ManLAM	Araf $\rightarrow$ <u>LGly</u>	< 10
Man-BSA	Man α $\rightarrow$ BSA	n.d.
ScMan	Man α (heterogeneous linkages) $\rightarrow$ <u>Gly</u>	< 10
AM	[Man α 1 $\rightarrow$ 2] _n Man α 1 $\rightarrow$ 5 Araf $\rightarrow$ <u>Gly</u>	< 10
Man ₃ -BSA	[Man α 1 $\rightarrow$ 2] ₂ Man α 1 $\rightarrow$ BSA	n.d.
Deacylated LAM	[Man α 1 $\rightarrow$ 2] _n Man α 1 $\rightarrow$ 5Araf $\rightarrow$ <u>Gly</u>	n.d.
(b) Structures		

(1000 RU $\approx$ 1 ng/mm²), a rough estimation indicates that the amount of lipoglycan bound to the surface is similar for LM and ManLAM, namely 6.9 and 6.5 fmol respectively (Figure 5).

In conclusion, we demonstrate here that immobilized hSP-A (i) bound preferentially to the mycobacterial lipoglycans and poorly to polysaccharides and (ii) interacted to the same extent with ManLAM and LM. Therefore these observations, consistent with our previous report [10], demonstrate that immobilization of hSP-A not only left the functionality of the protein intact, but also retained its selectivity for complex mycobacterial lipoglycans.

In order to verify the ManLAM structural motifs necessary for its recognition by immobilized hSP-A, we tested several structurally related compounds. As shown in Table 2, the LAM from *M. smegmatis*, naturally devoid of mannose caps (Table 2) and the *M. bovis* BCG ManLAM treated by an exo α -mannosidase, which removes the mannose capping [10], failed to bind to the immobilized hSP-A. Indeed, this demonstrates that the non-reducing terminal mannose residues capping the arabinan domain of ManLAM are absolutely required for the recognition of the ManLAM by hSP-A. However, the lack of interaction of Man-BSA or ScMan (Table 2) with the collectin strongly suggests that the presence of such terminal mannose motifs is not sufficient for recognition. It is likely that the unresponsiveness of these compounds results from the insufficient length of the mannose appendages of the Man-BSA (a single unit) or the heterogeneous mannose anomeric configurations and linkage types found in the mannan from *S. cerevisiae*. However, the mycobacterial ManAM also failed to interact (Table 2), despite a polysaccharidic structure similar to that of ManLAM, including the [Man α 1 $\rightarrow$ 2]_nMan α 1 $\rightarrow$ 5Araf ($n = 0-2$) capping motifs [20]. Thus the pre-

sence of these appendages is not sufficient to explain the exclusive binding of ManLAM to hSP-A. This assumption is verified by the unresponsiveness of synthetic BSA neo-glycoconjugates, also bearing the same α 1 $\rightarrow$ 2-mannose-linked trisaccharides (Man₃-BSA) (Table 2).

Thus, from these observations and the currently known structural differences between ManAM and ManLAM (Figure 1), the difference in the behaviour of these compounds in terms of recognition by hSP-A cannot be attributed to the structure of the saccharidic moiety, but rather to the presence of the ManLAM aglycone. The important role of the ManLAM phosphatidyl-*myo*-inositol anchor and, more precisely, of the lipids, is confirmed by the fact that the alkaline deacylation of ManLAM (d-ManLAM) abrogated the interaction with the collectins (Table 2).

Affinity of binding of ManLAM

A complex interaction kinetic between the immobilized hSP-A and ManLAM would support a direct interaction of the ManLAM hydrophobic aglycone with hSP-A, in addition to the carbohydrate-lectin interaction. Thus we analysed the real-time binding of ManLAM to hSP-A.

Injection of increasing concentrations of ManLAM over the immobilized hSP-A surface provided a dose-dependent response, consistent with a specific interaction. Preliminary analysis of the primary sensorgrams (Figure 6a) suggested a biphasic association process, with a rapid increase of the refractive index at the start of the injection followed by a lower association-phase rate. However, the respective blank curves obtained by injecting equal concentrations of analyte over a non-functionalized surface

Figure 6 Saturable binding of increasing concentrations of ManLAM to immobilized human SP-A

(a) Primary sensorgrams obtained by injection of increasing concentrations of ManLAM (50, 75, 100 and 125 $\mu\text{g/ml}$) across hSP-A functionalized (upper traces) and control chips (lower traces); (b) binding curves were calculated by subtracting control traces from the ManLAM-hSP-A sensorgrams. (c) Scatchard plot for the determination of the affinity of ManLAM binding to immobilized hSP-A [drawn from the R_{eq} (response at equilibrium) values calculated by non-linear curve fitting using the A+B type 1 or type 4 association models provided in the BIAevaluation 2.1 software].

(Figure 6a) indicated that the high initial *on*-rate constant observed corresponds to changes of the bulk refractive index upon injection of the analyte solution. Subtraction of these blank curves from the primary data yielded the specific binding sensorgrams depicted in Figure 6(b). When analysed by non-linear curve fitting using the BIAevaluation software, the association phases were best fitted to mono-exponential function describing a one-to-one interaction model. By contrast, the dissociation of ManLAM from the immobilized collectin was best fitted by a bi-exponential decay model, which indicates a more complex dissociation scheme. These biphasic dissociation curves may reflect a two-step dissociation process, probably resulting from further rebinding of ManLAM as the number of unoccupied binding sites increases during the course of the dissociation phase. The best way to verify the occurrence of such a rebinding process is to increase the analyte flow sufficiently (up to 50 $\mu\text{l/min}$) to minimize this effect. However, the low amount of highly purified lipoglycans available, unfortunately, does not

allow us to perform such experiments. Despite this limitation, using the BIAevaluation software, we estimated the kinetic parameters of the interaction (Table 3). As expected, both the *on*- and *off*-rates show almost no dependence on the concentration. This led to an averaged calculated apparent dissociation constant of ($K_D = k_{\text{off}}/k_{\text{on}}$ of 7.85×10^{-7} M) (Table 3). The K_D was also determined from a Scatchard analysis (Figure 6c). Since the binding equilibrium could not be reached during the 90 s injection period, the equilibrium response values are estimated by prospective calculation of the mono-exponential function used to fit the association phase. This approach leads to a similar K_D , namely 1.01×10^{-6} M. Thus, despite the complexity of the partners involved in the interaction, i.e. the octadecameric hSP-A and the structurally highly heterogeneous multivalent ManLAM, the appropriateness of the pseudo-first-order binding model is supported by the good convergence of the K_D values [calculated from the ratio of the kinetic constants ($k_{\text{off}}/k_{\text{on}}$) and estimated by Scatchard-plot analysis].

Role of the lipids

Because the association kinetics are in good agreement with the simplest $A + B \rightarrow AB$ association model, involving a single class of binding site, we hypothesize that the binding of the lipoglycans to the hSP-A is exclusively mediated by carbohydrate-lectin interactions. So it can be reasonably assumed that the lipids are responsible for the aggregation of the lipoglycans and that this process is required for the binding of the carbohydrate motifs to the hSP-A CRDs. This was further confirmed by the inhibiting effect of non-ionic detergent. Indeed, no interaction was observed when ManLAM was injected in the presence of a low concentration (0.005 %) of Polysorbate 20 (Tween 20) (results not shown). This result definitively demonstrates that the hydrophobic aglycone moiety of the lipoglycan is involved in the formation of supramolecular assemblies necessary for the multivalent binding of the largest number of CRDs of the collectin.

DISCUSSION

The first step of tuberculosis infection consists of the entry of the infectious bacilli into their target cells, namely alveolar macrophages. Unlike most bacteria, which are killed by macrophages, pathogenic mycobacteria are able to survive and even multiply in the cytoplasm of those 'professional phagocytes'. Indeed, mycobacteria survival depends on their ability to overcome the microbicidal power of the macrophages [31]. It is still controversial, but likely, that their entry path may provide mycobacteria with a survival advantage. Indeed, studies have demonstrated that the attachment of mycobacteria to the phagocytes varies according to the mycobacterial strain and the activation state of the phagocyte. It has been demonstrated that two key elements of innate immunity against airborne pathogens,

Table 3 Binding kinetic constants for the ManLAM-hSP-A interaction

Values are means $\pm$ S.E.M.

[ManLAM] [*] (μM)	$10^{-3} \times k_{\text{on}}^{\text{fast}}$ (s^{-1})	$10^{-3} \times k_{\text{off}}^{\text{slow}}$ (s^{-1})	$10^{-3} \times k_{\text{on}}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	$10^7 \times K_D$ (M) ($k_{\text{off}}^{\text{slow}}/k_{\text{on}}$)	Calculated $R_{\text{eq}}^{\dagger}$ (RU)
2.95 (50 $\mu\text{g/ml}$)	27.3 ± 0.9	1.52 ± 0.08	1.94 ± 0.072	7.8	97 ± 1.66
4.41 (75 $\mu\text{g/ml}$)	23.2 ± 0.8	1.34 ± 0.1	1.95 ± 0.125	6.9	104 ± 1.72
5.88 (100 $\mu\text{g/ml}$)	24.1 ± 0.5	1.75 ± 0.07	2.1 ± 0.084	8.3	110 ± 1.32
7.35 (125 $\mu\text{g/ml}$)	19.6 ± 0.4	1.60 ± 0.09	1.9 ± 0.079	8.4	114 ± 1.26

^{*} Averaged molecular mass of 17 kDa.

[†] Values calculated by non-linear curve fitting using the A+B type 1 or type 4 association model provided in the BIAevaluation 2.1 software.

pulmonary hSP-A and hSP-D, interact with *M. tuberculosis*. While the interaction of *M. tuberculosis* with the hSP-D reduces the uptake of the bacilli by macrophages [32], it has been shown that the interaction with hSP-A promotes this uptake [5]. Indeed, this suggests that the hSP-A may provide mycobacteria with an important target-cell entry pathway.

In a previous study we identified mycobacterial lipoglycans as putative ligands for the attachment of the hSP-A to mycobacteria. We also showed that the presence of terminal mannose residues and the aglycone moiety of the molecule were both required for optimal binding [10]. The presence of this hydrophobic component within the lipoglycans should, however, induce a supramolecular organization. It is, therefore, possible that this organization may be reflected by qualitative differences in terms of coating. The solid-phase presentation mode used in our previous study did not permit a full investigation of the role of the aglycone moiety. This is, however, an important step in the definition of the mycobacterial ligand of the hSP-A. To overcome this limitation, we developed a new binding micro-assay based on SPR. The first limiting step in the development of this assay was the immobilization of hSP-A. Indeed the physical and chemical characteristics of this protein make it particularly difficult to immobilize. Since disulphide bonds are required to maintain the correct folding of the CRDs, the thiol $\leftrightarrow$ disulphide exchange immobilization strategy recommended for acidic protein [24] must be avoided. Thus we attempted to immobilize hSP-A by coupling via the free amino groups of the ligand. This, however, requires electrostatic interaction to attract the ligand from the bulk solution close to the reactive groups of the matrix. The pH of the ligand working solution must be kept above the pK_a (≈ 3) of the dextran carboxy functions and 0.5–1 pH unit below the pI of the ligand. Despite the low pI of the hSP-A and its tendency to self-aggregate in acidic solutions, we were able to immobilize a significant amount of hSP-A by using a concentration of 100 μ g/ml and a pH of 3.5. Moreover, as demonstrated by repeated regeneration cycles and subsequent analysis, the immobilization is stable and preserves the activity of the CRDs of the collectin.

Indeed, we found that, among the mycobacterial surface glycoconjugates tested in the present study, the immobilized multimeric hSP-A only binds weakly to mycobacterial polysaccharides, but specifically recognizes the two major cell-wall lipoglycans, LM and ManLAM. This result, consistent with the results obtained in our previous study [10], shows that the immobilization of hSP-A does not modify the recognition specificity. We demonstrated that the binding of the hSP-A to immobilized lipoglycans results from the recognition of terminal mannopyranose residues. In LM, these are single mannose units $\alpha 1 \rightarrow 2$ -linked to a linear core of about 20 $\alpha 1 \rightarrow 6$ -linked manno-pyranose residues (Figure 1a) [22]. A similar repetitive structure, called the 'mannan core', is also present in the ManLAM and constitutes the inner moiety of the molecule. In addition to this mannan core, the ManLAM comprises a ramified arabinan domain partially capped by manno-oligosaccharides (one to three $\alpha 1 \rightarrow 2$ -linked mannose units) (Figure 1b). Digestion of ManLAM with α -exomannosidase completely abolished the interaction with immobilized hSP-A, confirming the critical role of the terminal mannose units of the ligand (Table 2). However, this treatment affects all the terminal mannose units – both those of the mannan core and those of the mannose caps. Therefore, to unambiguously identify the ManLAM mannose residues involved in the interaction, we tested PiLAM (phosphoinositol-capped lipoarabinomannan) – a structurally close lipoglycan produced by *M. smegmatis* [21]. This major antigen, found in the envelope of several non-pathogenic mycobacteria, only differs

from ManLAM by the presence of phosphoinositol appendages in place of the manno-oligosaccharide caps. As reported, this lipoglycan failed to interact with the immobilized hSP-A, despite the presence of a mannan domain closely related to that of the LM. Thus this result obviously indicates that the binding of the ManLAM to hSP-A is exclusively mediated by the manno-oligosaccharide caps, while the LM must interact through its branched single mannose units. The lack of binding of PiLAM can be attributed to steric hindrance resulting from the presence of the bulky arabinan domain preventing the recognition of the single mannose units branching from the mannan core.

The presence of terminal mannose residues is, however, not sufficient to ensure the interaction, as demonstrated by the low level of immobilized hSP-A binding to soluble ManAM – a naturally occurring mycobacterial cell-wall polysaccharide differing from the ManLAM only by the absence of the mannosylated phosphatidyl-*myo*-inositol anchor (Figure 1c). Moreover, deacylated ManLAM also failed to interact with the immobilized collectin. In conclusion, these results confirm that the aglycone moiety of the lipoglycan is absolutely necessary and plays an essential role in the recognition of the ligand terminal mannose by hSP-A.

From the literature and the known properties of both the ManLAM and hSP-A, two main hypotheses can be reasonably considered to explain the role of the lipidic moiety. The first is a direct interaction between the fatty acids and collectin. This is supported by the known propensity of the hSP-A to interact with phospholipids [33]. Such an interaction might contribute to the subsequent binding of the carbohydrate epitopes, by ensuring a preliminary lipid-mediated anchorage of the ligand to the protein, or by inducing some functionally essential conformational changes of the hSP-A. In contrast, the second hypothesis, supported by recent NMR analysis of these amphipathic molecules [34], postulates that the fatty acids are involved rather in lipoglycan aggregation or the formation of supramolecular assemblies. This organization would then allow a higher-affinity binding to hSP-A, due to the possibility of establishing a multivalent interaction with the multiple CRDs of the collectin [11].

The analysis of the association phase of the sensorgrams recorded for different ManLAM concentrations are best fitted by a mono-exponential interaction model, which obviously supports a binding mechanism exclusively mediated by carbohydrates, excluding any other putative lipid–protein interactions. By contrast, the dissociation phases of the sensorgrams are best fitted by a bi-exponential decay model. This suggests a more complicated dissociation mechanism, which may be attributed to the rebinding of the analyte during the dissociation phase. Such biphasic decay plots are generally observed when the analyte is aggregated or self-organized in solution [35]. Furthermore, the absence of interaction in presence of detergent also suggests that a particular self-organization of the lipoglycans in solution is necessary for their recognition by hSP-A. Indeed, we hypothesize that hSP-A recognition of mycobacterial lipoglycans is restricted by their ability to self-associate to form larger supramolecular assemblies, thereby allowing multivalent interactions with the largest number of CRDs of the hSP-A molecule [36]. Thus it can be reasonably suspected that the selection of hSP-A high-affinity ligands is controlled by their structure. Indeed, the ligand structure has to match the stringent structural requirements, in terms of anomeric configuration, linkage type and number of linked residues, for high-affinity binding to an extended site such as described for the MBP CRDs [36,37].

According to the supramolecular-organization hypothesis, and from a practical point of view, it is obvious that the strategy we

and others [12] used to search for potential targets of hSP-A may have led exclusively to the identification of glycosylated compounds, which, once purified, have the ability to self-organize and/or mimic an organization state similar to that of the micro-organism's cell surface. Moreover, this may explain the limited number of purified ligands of hSP-A identified to date – the Gram-negative lipopolysaccharides [12], the mycobacterial lipoglycans [10] and the *Pneumocystis carinii* major surface glycoproteins [15]. Consequently, care must be taken regarding the possible conclusions of our results, that is, the lack of interaction between purified ManAM and hSP-A does not exclude the possibility that this polysaccharide can be recognized when embedded in the mycobacterial cell wall. It is noteworthy that, among the mycobacterial lipoglycans, ManLAM is of special interest, due to its numerous biological activities and potential involvement in the pathophysiological disorders seen during the course of tuberculosis infections [38]. In this regard, the organizing role of the ManLAM fatty acids in inducing supramolecular assemblies should be taken into account when considering its immunomodulatory activities and, more specifically, those explicitly reported as being dependent on the presence of the lipidic anchor [19].

Finally, our results also afford some insights into the understanding of the basic molecular mechanism directing the immune discrimination of self and non-self by the collectins. Indeed, their particular complex structure may be seen as the result of an evolutionary process that has selected molecules more particularly specialized in the recognition of a broad range of pathogen-associated molecular patterns (PAMPs) [39]. These PAMPs, present on the surface of several groups of pathogenic micro-organisms, result from the repetition of a small number of target motifs, such as mannose residues. These residues are borne by different classes of genus-specific compounds such the lipopolysaccharides of Gram-negative bacteria, the mannans of yeast, the lipoteichoic acids of Gram-positive bacteria or the glycolipids and lipoglycans of mycobacteria. By contrast, such compounds are likely to be absent from higher-organism cell surfaces. Indeed, although present, the mannose units found on the surface of mammalian cells not only do not share such a repetitive structural organization, but they are also much less accessible due to the masking presence of additional carbohydrate residues not recognized by collectins, such as galactose or sialic acid. The binding of hSP-A to PAMP-decorated pathogens relies on a complex double-level mechanism involving first, at the basic molecular level, the interaction of individual lectin domains with terminal mannose units widely found on the surface of the invading micro-organism. The multimeric association of several CRDs introduces a higher level of complexity for the specific recognition of non-self. Indeed the high-affinity multivalent binding of foreign particles depends on the repetition density and the arrangement of the target residues (mannose units) which may fulfil the geometric conditions imposed by the spatial organization of the multiple lectin domains of the hSP-A [36].

We thank Dr Mitchell Kronenberg (La Jolla Institute for Allergy and Immunology, San Diego, CA, U.S.A.) for critical reading of the manuscript before its submission. We also thank Dr J. Nigou (I.P.B.S.) for providing mycobacterial lipoglycans and Professor P. Rougé (I.P.B.S.) for skilful advice concerning SPR technology and data analysis. This work was supported by grants from the Mission Scientifique et Technique du Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche (ACC SV6 9506005 and ACC SV14 1411587) and from the Région Midi-Pyrénées (RECH/9702343). S.S. is a recipient of a doctoral fellowship from the French Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche.

REFERENCES

- Holmskov, U., Malhotra, R., Sim, R. B. and Jensenius, J. C. (1994) Collectins: collagenous C-type lectins of the innate immune defense system. *Immunol. Today* **15**, 67–74
- Crouch, E., Hartshorn, K. and Ofek, I. (2000) Collectins and pulmonary innate immunity. *Immunol. Rev.* **173**, 52–65
- Lawson, P. R. and Reid, K. B. (2000) The roles of surfactant proteins A and D in innate immunity. *Immunol. Rev.* **173**, 66–78
- Tino, M. J. and Wright, J. R. (1996) Surfactant protein A stimulates phagocytosis of specific pulmonary pathogens by alveolar macrophages. *Am. J. Physiol.* **270**, L677–L688
- Downing, J. F., Pasula, R., Wright, J. R., Twigg, H. L. D. and Martin, W. J. D. (1995) Surfactant protein A promotes attachment of *Mycobacterium tuberculosis* to alveolar macrophages during infection with human immunodeficiency virus. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4848–4852
- Gaynor, C. D., McCormack, F. X., Voelker, D. R., McGowan, S. E. and Schlesinger, L. S. (1995) Pulmonary surfactant protein A mediates enhanced phagocytosis of *Mycobacterium tuberculosis* by a direct interaction with human macrophages. *J. Immunol.* **155**, 5343–5351
- Chroneos, Z. C., Abdolrasulnia, R., Whitsett, J. A., Rice, W. R. and Shepherd, V. L. (1996) Purification of a cell-surface receptor for surfactant protein A. *J. Biol. Chem.* **271**, 16375–16383
- McCormack, F. X. (1998) Structure, processing and properties of surfactant protein A. *Biochim. Biophys. Acta* **1408**, 109–131
- Haagsman, H. P., Hawgood, S., Sargeant, T., Buckley, D., White, R. T., Drickamer, K. and Benson, B. J. (1987) The major lung surfactant protein, SP 28–36, is a calcium-dependent, carbohydrate-binding protein. *J. Biol. Chem.* **262**, 13877–13880
- Sidobre, S., Nigou, J., Puzo, G. and Rivière, M. (2000) Lipoglycans are putative ligands for the human pulmonary surfactant protein A attachment to mycobacteria: critical role of the lipids for lectin–carbohydrate recognition. *J. Biol. Chem.* **275**, 2415–2422
- Weis, W. I., Drickamer, K. and Hendrickson, W. A. (1992) Structure of a C-type mannose-binding protein complexed with an oligosaccharide. *Nature (London)* **360**, 127–134
- Van Iwaarden, J. F., Pikaar, J. C., Storm, J., Brouwer, E., Verhoef, J., Oosting, R. S., van Golde, L. M. and van Strijp, J. A. (1994) Binding of surfactant protein A to the lipid A moiety of bacterial lipopolysaccharides. *Biochem. J.* **303**, 407–411
- Kabha, K., Schmegner, J., Keisari, Y., Parolis, H., Schlepper-Schaeffer, J. and Ofek, I. (1997) SP-A enhances phagocytosis of *Klebsiella* by interaction with capsular polysaccharides and alveolar macrophages. *Am. J. Physiol.* **272**, L344–L352
- McNeely, T. B. and Coonrod, J. D. (1994) Aggregation and opsonization of type A but not type B *Hemophilus influenzae* by surfactant protein A. *Am. J. Respir. Cell. Mol. Biol.* **11**, 114–122
- Zimmerman, P. E., Voelker, D. R., McCormack, F. X., Paulsrud, J. R. and Martin, W. J. D. (1992) 120-kD surface glycoprotein of *Pneumocystis carinii* is a ligand for surfactant protein A. *J. Clin. Invest.* **89**, 143–149
- Weikert, L. F., Edwards, K., Chrones, Z. C., Hager, C., Hoffman, L. and Shepherd, V. L. (1997) SP-A enhances uptake of bacillus Calmette-Guérin by macrophages through a specific SP-A receptor. *Am. J. Physiol.* **272**, L989–L995
- Pasula, R., Downing, J. F., Wright, J. R., Kachel, D. L., Davis, Jr, T. E. and Martin, W. J. D. (1997) Surfactant protein A (SP-A) mediates attachment of *Mycobacterium tuberculosis* to murine alveolar macrophages. *Am. J. Respir. Cell. Mol. Biol.* **17**, 209–217
- Astarié-Dequeker, C., N'Diaye, E. N., Le Cabec, V., Rittig, M. G., Prandi, J. and Maridonneau-Parini, I. (1999) The mannose receptor mediates uptake of pathogenic and nonpathogenic mycobacteria and bypasses bactericidal responses in human macrophages. *Infect. Immun.* **67**, 469–477
- Nigou, J., Gilleron, M., Cahuzac, B., Bounery, J. D., Herold, M., Thurnher, M. and Puzo, G. (1997) The phosphatidyl-*myo*-inositol anchor of the lipoarabinomannans from *Mycobacterium bovis* bacillus Calmette Guérin. Heterogeneity, structure, and role in the regulation of cytokine secretion. *J. Biol. Chem.* **272**, 23094–23103
- Nigou, J., Gilleron, M., Brando, T., Vercellone, A. and Puzo, G. (1999) Structural definition of arabinomannans from *Mycobacterium bovis* BCG. *Glycoconj. J.* **16**, 257–264
- Gilleron, M., Himoudi, N., Adam, O., Constant, P., Venisse, A., Riviere, M. and Puzo, G. (1997) *Mycobacterium smegmatis* phosphoinositols-glycoarabinomannans. Structure and localization of alkali-labile and alkali-stable phosphoinositides. *J. Biol. Chem.* **272**, 117–124
- Gilleron, M., Nigou, J., Cahuzac, B. and Puzo, G. (1999) Structural study of the lipomannans from *Mycobacterium bovis* BCG: characterisation of multiacylated forms of the phosphatidyl-*myo*-inositol anchor. *J. Mol. Biol.* **285**, 2147–2160
- Wright, J. R., Wager, R. E., Hawgood, S., Dobbs, L. and Clements, J. A. (1987) Surfactant apoprotein *M*₂ = 26 000–36 000 enhances uptake of liposomes by type II cells. *J. Biol. Chem.* **262**, 2888–2894

- 24 Johnsson, B., Lofas, S., Lindquist, G., Edstrom, A., Muller Hillgren, R. M. and Hansson, A. (1995) Comparison of methods for immobilization to carboxymethyl dextran sensor surfaces by analysis of the specific activity of monoclonal antibodies. *J. Mol. Recognit.* **8**, 125–131
- 25 Doyle, I. R., Davidson, K. G., Barr, H. A., Nicholas, T. E., Payne, K. and Pfitzner, J. (1998) Quantity and structure of surfactant proteins vary among patients with alveolar proteinosis. *Am. J. Respir. Crit. Care Med.* **157**, 658–664
- 26 Ruano, M. L., Garcia-Verdugo, I., Miguel, E., Perez-Gil, J. and Casals, C. (2000) Self-aggregation of surfactant protein A. *Biochemistry* **39**, 6529–6537
- 27 Stenberg, E., Persson, B., Roos, H. and Urbaniczki, J. (1990) Quantitative determination of surface concentration of protein with surface plasmon resonance using radiolabeled proteins. *J. Colloid Interface Sci.* **43**, 513–526
- 28 Daffe, M., McNeil, M. and Brennan, P. J. (1993) Major structural features of the cell wall arabinogalactans of *Mycobacterium*, *Rhodococcus*, and *Nocardia* spp. *Carbohydr. Res.* **249**, 383–98
- 29 Lemassu, A. and Daffe, M. (1994) Structural features of the exocellular polysaccharides of *Mycobacterium tuberculosis*. *Biochem. J.* **297**, 351–357
- 30 Venisse, A., Berjeaud, J. M., Chaurand, P., Gilleron, M. and Puzo, G. (1993) Structural features of lipoarabinomannan from *Mycobacterium bovis* BCG. Determination of molecular mass by laser desorption mass spectrometry. *J. Biol. Chem.* **268**, 12401–12411
- 31 Kaufmann, S. H. (2001) How can immunology contribute to the control of tuberculosis? *Nat. Immunol.* **1**, 20–30
- 32 Ferguson, J. S., Voelker, D. R., McCormack, F. X. and Schlesinger, L. S. (1999) Surfactant protein D binds to *Mycobacterium tuberculosis* bacilli and lipoarabinomannan via carbohydrate–lectin interactions resulting in reduced phagocytosis of the bacteria by macrophages. *J. Immunol.* **163**, 312–321
- 33 Ross, G. F., Notter, R. H., Meuth, J. and Whitsett, J. A. (1986) Phospholipid binding and biophysical activity of pulmonary surfactant-associated protein (SAP)-35 and its non-collagenous COOH-terminal domains. *J. Biol. Chem.* **261**, 14283–14291
- 34 Nigou, J., Gilleron, M. and Puzo, G. (1999) Lipoarabinomannans: characterization of the multiacylated forms of the phosphatidyl-*myo*-inositol anchor by NMR spectroscopy. *Biochem. J.* **337**, 453–460
- 35 Garcia, K. C., Radu, C. G., Ho, J., Ober, R. J. and Ward, E. S. (2001) Kinetics and thermodynamics of T cell receptor–autoantigen interactions in murine experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 6818–6823
- 36 Hoffmann, J. A., Kafatos, F. C., Janeway, C. A. and Ezekowitz, R. A. (1999) Phylogenetic perspectives in innate immunity. *Science* **284**, 1313–1318
- 37 Weis, W. I. and Drickamer, K. (1996) Structural basis of lectin–carbohydrate recognition. *Annu. Rev. Biochem.* **65**, 441–473
- 38 Chatterjee, D. and Khoo, K. H. (1998) Mycobacterial lipoarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects. *Glycobiology* **8**, 113–120
- 39 Medzhitov, R. and Janeway, Jr, C. (2000) Innate immunity. *N. Engl. J. Med.* **343**, 338–344

Received 12 November 2001/27 February 2002; accepted 22 April 2002