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A mycobacterial lipoarabinomannan specific monoclonal antibody and its F(ab′)$_2$ fragment prolong survival of mice infected with *Mycobacterium tuberculosis*

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SUMMARY

Lipoarabinomannan (LAM) is a major structural carbohydrate antigen of the outer surface of *Mycobacterium tuberculosis*. High antibody titres against LAM are often seen in active tuberculosis (TB). The role of such LAM-specific antibodies in the immune response against TB is unknown. Here we have investigated a monoclonal antibody (MoAb) SMITB14 of IgG1 subclass and its corresponding F(ab′)$_2$ fragment directed against LAM from *M. tuberculosis* strain H37Rv. MoAb SMITB14 was shown by immunofluorescence to bind to whole cells of the clinical isolate *M. tuberculosis* strain Harlingen as well as to *M. tuberculosis* H37Rv. The binding of MoAb SMITB14 to LAM was inhibited by arabinomannan (AM) and oligosaccharides (5·2 kDa) derived from LAM, showing that the MoAb binds specifically to the AM carbohydrate portion of LAM. In passive protection experiments BALB/c mice were infected intravenously with *M. tuberculosis* Harlingen. MoAb SMITB14 was added intravenously either prior to, or together with, the bacteria. The antibody proved to be protective against the *M. tuberculosis* infection in terms of a dose-dependent reduction in bacterial load in spleens and lungs, reduced weight loss and, most importantly, increased long-term survival.

Keywords lipoarabinomannan monoclonal antibody passive protection tuberculosis

INTRODUCTION

In many bacterial infections antibodies directed against bacterial carbohydrate surface antigens have been shown to be important in protective immunity. However, immunity against tuberculosis (TB) has been assumed to rely solely on cellular defence mechanisms, and antibody-mediated immunity has either been disregarded or even thought to facilitate the development of active TB. These assumptions were based mainly on the results of early serum therapy studies performed in the beginning of the 1900s and on serological investigations, of various clinical forms, of TB in humans and experimental animals, including passive protection experiments using immune sera (for a recent extensive review see [1]).

In these earlier studies the protective effect of antibodies against *Mycobacterium tuberculosis* and its target antigens were not, or only poorly, characterized. There is some early evidence that antibodies directed towards mycobacterial carbohydrate antigens such as lipoarabinomannan (LAM) may be beneficial [1], while there is less evidence of beneficial effects conferred by antibodies directed against protein antigens, including the components comprising purified protein derivative (PPD). Hence, Choucroun reported in 1949 a correlation between the presence of serum antibodies to *M. tuberculosis* carbohydrate antigens and acquired immunity to disease [2]. Later, studies by Sibert *et al*. associated protective effects with antibodies to mycobacterial polysaccharides but not to protein antigens [3,4]. Recently, Costello *et al*. found that children with disseminated TB had a significantly lowered IgG response to mycobacterial LAM and other mycobacterial antigens [5]. They concluded that a weak antibody response to LAM and other mycobacterial antigens before or in the early stages of infection increased the likelihood of dissemination.

LAM is a major structural carbohydrate component of the outer surface of *M. tuberculosis*, and frequently gives rise to high antibody responses in the infected host [6]. The role of such antibodies in the pathogenesis of TB is not known. By using monoclonal antibodies (MoAbs) it is possible to study antibodies with precisely defined epitope specificity, subclass and isotype for their possible beneficial or deleterious activity in TB.

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We have prepared an array of MoAbs with specificity for epitopes located in the arabinomannan part of LAM. Here we describe the preparation of MoAbs and the effect of one of these MoAbs of IgG1 subclass and its corresponding $F(ab')_2$ fragment in passive protection experiments in BALB/c mice against experimental TB.

MATERIALS AND METHODS

Bacterial strains

M. tuberculosis H37Rv, obtained originally from the ATCC collection, was maintained at the Swedish Institute for Infectious Disease Control (SIIDC), Stockholm, Sweden. The clinical isolate *M. tuberculosis* strain Harlingen used for the experimental infections was kindly provided by Dr J. van Embden, RIVM, the Netherlands.

Materials and reagents

A mouse MoAb of IgG1 subclass against human haemoglobin (SMIHHG1) was obtained from the Department of Immunology, Karolinska Institute, Stockholm. Microtitre Maxisorb™ 96-well plates were from Nunc, Kampstrup, Denmark. Yeast mannan, methyl α-D-mannopyranoside, DL-arabinose, pepsin and alkaline phosphatase-conjugated goat antimouse IgG were from Sigma Chemicals, USA. If not stated otherwise, all other chemicals were of analytical purity.

Preparation of LAM

LAM from *M. tuberculosis* H37Rv was prepared as described previously [7]. Arabinomannan (AM) was obtained by mild alkaline hydrolysis of LAM as described previously [8]. Sodium periodate oxidation of delipidated purified LAM was done by treatment with 10 mM $NaIO_4$ in acetate buffer pH 6·0 at + 4°C in the dark for 15 min.

Preparation of anti-LAM monoclonal antibodies

The antigen for immunizations was prepared by mixing 1 mg of purified LAM from *M. tuberculosis* H37Rv with 4 mg of heat-killed *M. tuberculosis* H37Rv bacteria. The mixture was evaporated slowly to dryness under reduced pressure and resuspended in 5 ml phosphate buffered saline (PBS). Ten outbred female Naval Medical Research Institute (NMRI) mice (8–10 weeks, B & K, Sweden) were injected, intraperitoneum, with 200 μl of the antigen mixture emulsified in complete Freund's adjuvant (Difco Laboratory, USA) at a ratio of 1 : 1. The mice were then boosted twice, at 2-week intervals, with the same amount of antigen in incomplete Freund's adjuvant (Difco Laboratory). Three days prior to spleen cells harvest for hybridoma production, the mice were given 200 μl of the antigen intravenously in PBS without adjuvant. Spleen cells were fused with SP2/AG64G14 myeloma cells essentially following the method of Köhler and Milstein [9]. Hybridomas that secreted LAM-specific antibodies were identified by enzyme-linked immunosorbent assay (ELISA) and cloned three times by limiting dilutions. Individual colonies were then chosen for expansion. The MoAbs were purified, from culture supernatants, by affinity chromatography over protein G-Sepharose according to the supplier's instructions (GamMAbind Plus Sepharose, Pharmacia & Upjohn, Uppsala, Sweden). The clonal purity of the purified MoAbs was ascertained by isoelectric focusing using automated PhastSystem™ with PhastGel IEF 3–9 (Pharmacia & Upjohn, Uppsala, Sweden; DT File no. 210). After focusing, the gels were silver-stained as recommended by the manufacturer (ST file no. 100).

The isotypes of the MoAbs were identified by ELISA, using LAM as coating antigen and alkaline phosphatase-conjugated goat antimouse IgG subclass specific antibodies (Sigma Chemical Co, USA). One MoAb (SMITB14) of IgG1 subclass was chosen for further studies of its potential effect in passive protection experiments.

Quantification of MoAbs

The relative titres of the MoAbs were determined by ELISA. Wells of polystyrene microplates (Maxisorb, Nunc, Denmark) were coated with 100 μl of purified LAM (10 μg/ml) in 0·05 M carbonate buffer, pH 9·6, at room temperature overnight. The plates were washed three times with rinsing buffer (PBS containing 0·05% Tween), and then blocked with 0·5% casein for 1 h at 37°C. After washing, 100 μl of serial dilutions of each MoAb were added to the wells and incubated for 1 h at 37°C. After washing with rinsing buffer, 100 μl of alkaline phosphatase-conjugated goat, antimouse IgG (Sigma Chemical Co., diluted 1/2000 in PBS) was added to each well and the plates were incubated for a further 1 h at 37°C. After subsequent washings, the plates were developed at room temperature using p-nitrophenyl phosphate (Sigma Chemical Co.) as substrate and the colour reaction was measured by increase in absorbance at 405 nm using an ELISA reader (Dynatech, MR 5000).

Preparation of $F(ab')_2$ fragments

$F(ab')_2$ fragments from MoAb SMITB14 and from the isotype control MoAb SMIHHG1 were prepared as follows: MoAbs were dialysed for 6 h at room temperature against 0·1 M glycine-HCl buffer, pH 2·8, followed by a second dialysis against 0·1 M Na-acetate buffer, pH 4·5, overnight. $F(ab')_2$ fragments were prepared by treating the antibody with 1% pepsin (w/w) in the acetate buffer for 2·5 h at 37°C. The reaction was stopped by raising the pH to 8·0 with 0·1 M NaOH, and the resulting $F(ab')_2$ fragments were purified by ultrafiltration using an Omega ultrafiltration cell with a 50 kDa MW cut-off (Filtron, Northborough, USA). The purity of the obtained $F(ab')_2$ fragments was ascertained by sodium dodecyly suplohate-polyacrylamide gel electrophoresis (SDS-PAGE) (4–15% Phast Gels™ Pharmacia, Uppsala, Sweden) under reducing and nonreducing conditions.

Binding specificity of MoAb SMITB14

The ability of MoAb SMITB14 to detect whole mycobacterial cells was examined by immunofluorescence, as described previously [10]. Briefly, 20 μl of heat-killed bacteria in PBS (10^5/ml) was gently mixed with 20 μl of fluoroscein isothyocyanate (FITC)-labelled MoAb in PBS (0·2 mg/ml). Binding was detected using an immunofluorescence microscope.

The specificity of MoAb SMITB14 was established by inhibition ELISA. ELISA was performed as described above, but prior to the addition to the wells, the MoAb SMITB14 was preincubated for 30 min at room temperature with different inhibitors at varying concentrations.

Experimental infections

Female BALB/c mice (8–10 weeks, B&K, Stockholm, Sweden), were used throughout these studies. After delivery to the BSL3 laboratory at SIIDC the mice were acclimatized for 1 week in a BSL3 isolator (Elwyn E. Roberts Isolators Ltd, Shropshire, UK). Mice were housed at a maximum of five animals per cage.

M. tuberculosis Harlingen was cultured on solid Löwenstein Jensen medium for 4 weeks and then propagated in liquid Middlebrook 7H9 medium for another 3 weeks. Aliquots of bacilli were suspended in 7H9 medium with 10% glycerol at 10^7–10^8 bacilli/ml and frozen at −70°C. The viability of the frozen suspension was checked by plating the thawed bacteria on Middlebrook 7H11 agar supplemented with oleic acid albumin–dextrose–catalase (OADC; Difco).

Prior to infection, frozen ampoules of *M. tuberculosis* Harlingen were thawed, diluted in PBS to the appropriate concentration and shaken on a Vortex shaker for 10 s to disperse clumps of bacteria. The titre of the inoculum was reconfirmed by dilution plating of the bacterial stock. Mice were infected intravenously (i.v.) via the lateral tail vein.

In the passive protection experiments the respective MoAbs and $F(ab')_2$ fragments were diluted in PBS and mixed with *M. tuberculosis* Harlingen at indicated concentrations. The mixture was incubated for 30 min with constant agitation at room temperature and 100 μl of the mixture was injected in the lateral tail vein. In some experiments MoAbs (100 μl) were administered i.v. 60 min prior to the injection of bacteria (100 μl). For intranasal (i.n.) infection experiments, the $F(ab')_2$ fragment of MoAb SMITB14 was mixed with freshly thawed *M. tuberculosis* Harlingen to which Lutrol® F127 was added (BASF Ludwigshafen, Germany; final concentration 0·12%) to give a single cell suspension. After 15 min incubation at room temperature 30 μl of such mixture was administered through both nostrils (3×5 μl per nostril) with the animal held in an upright position.

The mice were observed daily and weighed individually on the day of infection and then once a week. Mice were sacrificed at indicated times after infection, or when moribund, by cervical neck dislocation and lungs, spleens and livers were aseptically removed, weighed and homogenized in a Colworth Stomacher 80 homogenizer (A. J. Seward UAC House, Blackfriar Road, London) in 0·01% Tween 80-saline solution. Colony-forming units (CFU) were then determined by plating the appropriate dilutions of the homogenates on Middlebrook 7H11 agar supplemented with OADC, polymyxin B and amphotericin B.

Statistical analysis

Data were analysed using the unpaired two-tailed Student's *t*-test for CFU counts and Kaplan–Meier survival plots followed by the log-rank test for survival data.

RESULTS

Characterization of MoAb SMITB14

FITC-labelled MoAb SMITB14 efficiently bound to whole *M. tuberculosis* bacteria (H37Rv and Harlingen strains) as well as to the attenuated *M. bovis* Calmette–Guérin strain (BCG) but not to other non-mycobacterial species such as *Escherichia coli* strains of various O serotypes, *Streptococcus pneumoniae* isolates, strains of Salmonella, *Yersinia enterocolitica* and *Y. pseudotuberculosis* and *Nocardia* sp. (data not shown). The specificity of MoAb SMITB14 was analysed in more detail in ELISA inhibition experiments, using the following potential inhibitors: AM, yeast mannan, methyl α-D-mannopyranoside and DL-arabinose (Fig. 1). The binding of MoAb SMITB14 to LAM was inhibited by purified AM in a dose-dependent manner, while yeast mannan, methyl α-D-mannopyranoside and DL-arabinose at a concentration up to 40 μg/ml were not inhibitory. The inhibition by AM was abolished completely after sodium periodate oxidation, further confirming that specificity of MoAb SMITB14 binding resided in the carbohydrate portion of LAM (data not shown).

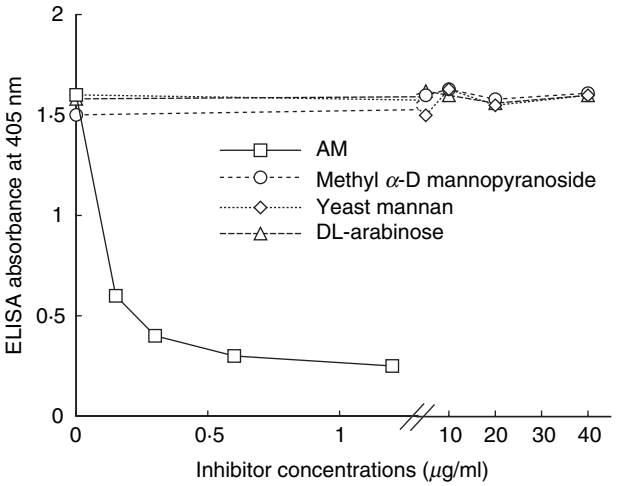

Fig. 1. Inhibition of binding of MoAb SMITB14 to LAM (10 μg/ml as coating antigen) assayed by ELISA. The following putative inhibitors, arabinomannan (AM) at a concentration ranging from 0 to 1 μg/ml, mannan, methyl a-D-mannopyranoside and DL-arabinose at concentrations of 0–40 μg/ml were preincubated with the appropriate dilution of the MoAb.

Effect of MoAb SMITB14 on bacterial loads in organs of i.v.-infected mice

In a first experiment (Fig. 2, exp. I) BALB/c mice were challenged i.v. with *M. tuberculosis* Harlingen, either alone or in combination with MoAb SMITB14. After 14 days the mice were sacrificed and the CFU counts in lungs and spleens were determined. In mice given bacteria with MoAb SMITB14 there was a dose-dependent reduction in CFU counts in both lungs and spleens, compared to mice given bacteria alone. Thus, at the highest concentration of MoAb SMITB14 (1 mg per mouse) the difference in bacterial counts reached 1·8 log unit and 1·3 log unit for lungs and spleens, respectively. These differences were, however, non-significant due to the considerable variation between individual animals.

These results encouraged us to perform a second experiment (Fig. 2, exp. II). Mice were challenged i.v. with *M. tuberculosis* Harlingen alone or in combination with $F(ab')_2$ fragment of MoAb SMITB14 and sacrificed 14 days later. The CFU counts were significantly ($P = 0{\cdot}02$) lower in the spleens of mice given the $F(ab')_2$ fragments and also lower in the lungs.

In yet another experiment (Fig. 2, exp. III) we attempted to determine whether a similar protective effect could be achieved by systemic administration of MoAb prior to challenge with *M. tuberculosis* . Therefore, one group of mice was given a mixture of bacteria and MoAb SMITB14 and another group of mice was inoculated with MoAb SMITB14 1 h prior to *M. tuberculosis* infection. Control groups consisted of mice that received an irrelevant MoAb of the matched isotype (SMIHHG1, IgG1) either together with, or prior to, bacteria. All mice were sacrificed 2 days after infection. In both groups of mice which received MoAb SMITB14 there was significant reduction of bacterial loads in lungs, livers and spleens compared to the mice which received isotype control MoAb (Fig. 2, exp. III). This difference was of a similar order of magnitude in mice injected

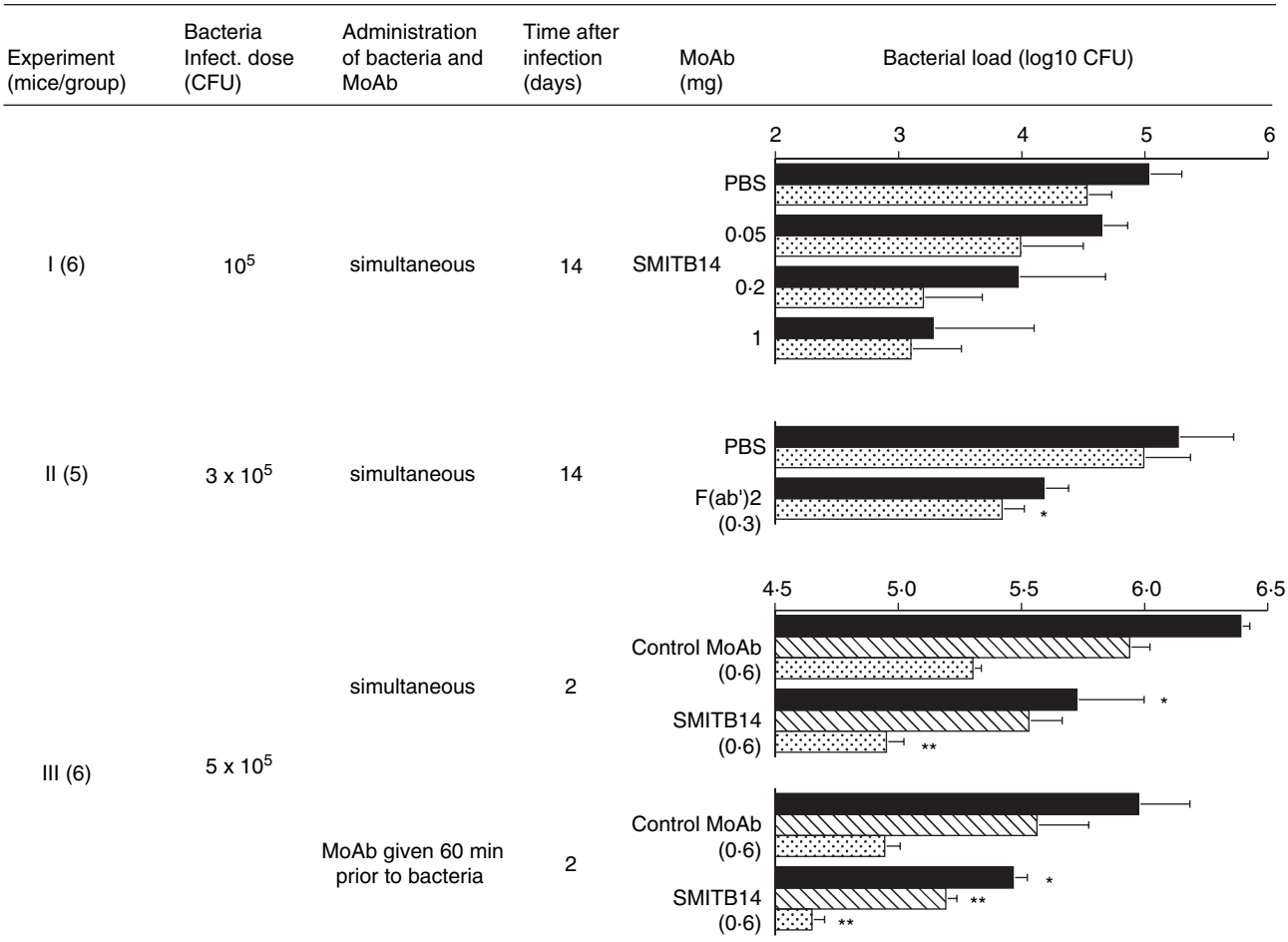

Fig. 2. Bacterial counts in organs of BALB/c mice after i.v. infection with *M. tuberculosis* strain Harlingen. CFU counts were determined in different organs: black bars, lung; dotted bars, spleen; striated bars, liver. Mean $\log_{10}$ (CFU) ± s.e.m. from five to six mice per group are shown. Asterisks indicate statistically significant difference between control and specific MoAb group (*$P < 0{\cdot}05$; **$P < 0{\cdot}01$). Experiment I: mice were challenged i.v. with 10^5 CFU *M. tuberculosis* Harlingen, either alone, or in combination with indicated amounts of MoAb SMITB14. After 14 days the mice were sacrificed and the CFU counts in lungs and spleens were determined. Experiment II: mice were challenged i.v. with 3×10^5 CFU *M. tuberculosis* Harlingen alone or in combination with $F(ab')_2$ fragment of MoAb SMITB14 (0·3 mg) and sacrificed 14 days later and CFU counts in lungs and spleens were determined. Experiment III: one group of mice was given a mixture of bacteria and MoAb SMITB14 (5×10^5 CFU *M. tuberculosis* Harlingen and 0·6 mg MoAb) and another group of mice was inoculated with MoAb SMITB14 1 h prior to *M. tuberculosis* infection. Control groups consisted of mice that received an irrelevant MoAb of the same isotype as SMITB14 (SMIHHG1, IgG1, directed against human haemoglobin) at the same dose, either together with or prior to bacteria. All mice were sacrificed 2 days after infection and the CFU counts in lungs and spleens were determined.

with bacteria–MoAb mixture and mice injected with MoAb prior to bacteria. Furthermore, the organs of mice that received MoAb SMITB14 before infection showed lower bacterial loads, relative to the mice that were given the same MoAb and bacteria together.

In order to confirm that the reduction in CFU counts observed in organs of challenged mice was not due to a direct killing or aggregation of bacteria by MoAb SMITB14, the aliquots of the inoculates used for infections consisting of bacteria alone, or mixtures of bacteria and MoAb SMITB14 or its $F(ab')_2$ fragment, were plated on Middlebrook agar. There was no reduction in CFU counts in the presence of the MoAb or the $F(ab')_2$ fragment, as compared to bacteria alone or bacteria mixed with the isotype control MoAb (data not shown).

Effect of MoAb SMITB14 on body weight and survival of i.v.-infected mice

Fourteen days postchallenge there was a significant ($P = 0{\cdot}02$) reduction in weight in the group of mice given *M. tuberculosis* alone compared to the mice given *M. tuberculosis* in combination with $F(ab')_2$ fragment (Fig. 3a, exp. I). A similar observation was made when the experiment was repeated using a somewhat higher natio of SMITB14 or its $F(ab')_2$ fragment to bacteria (Fig. 3b). Mice receiving bacteria together with MoAb or $F(ab')_2$ fragment suffered significantly less weight loss ($P = 0{\cdot}024$ and $P = 0{\cdot}006$, respectively) than mice infected with bacteria alone (Fig. 3b).

The possible effect of passive immunization with MoAb SMITB14 on long-term survival of mice was studied next. Mice

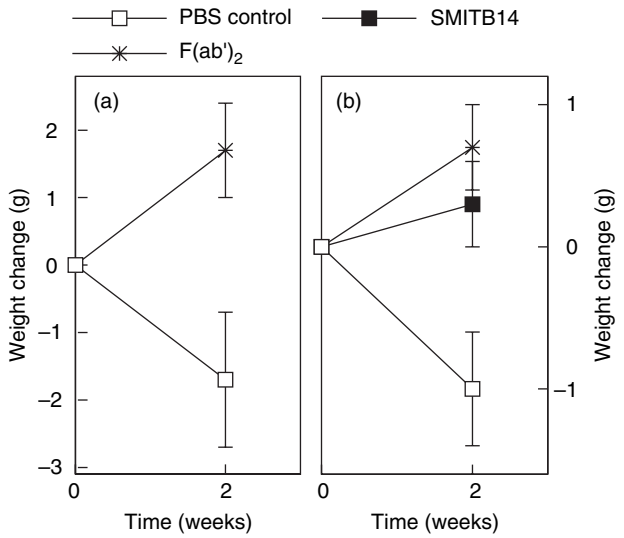

Fig. 3. Change in body weight of BALB/c mice 2 weeks after intravenous infection with *M. tuberculosis* Harlingen administered alone, with MoAb SMITB14 or the corresponding F(ab′)$_2$ fragment. (a) Harlingen, 3×10^5 CFU; F(ab′)$_2$, 0·4 mg. (b) Harlingen, 10^5 CFU; SMITB14, 0·4 mg, and F(ab′)$_2$, 0·45 mg. Mean weight ± s.e.m. of five mice per group is shown. For details see Materials and methods.

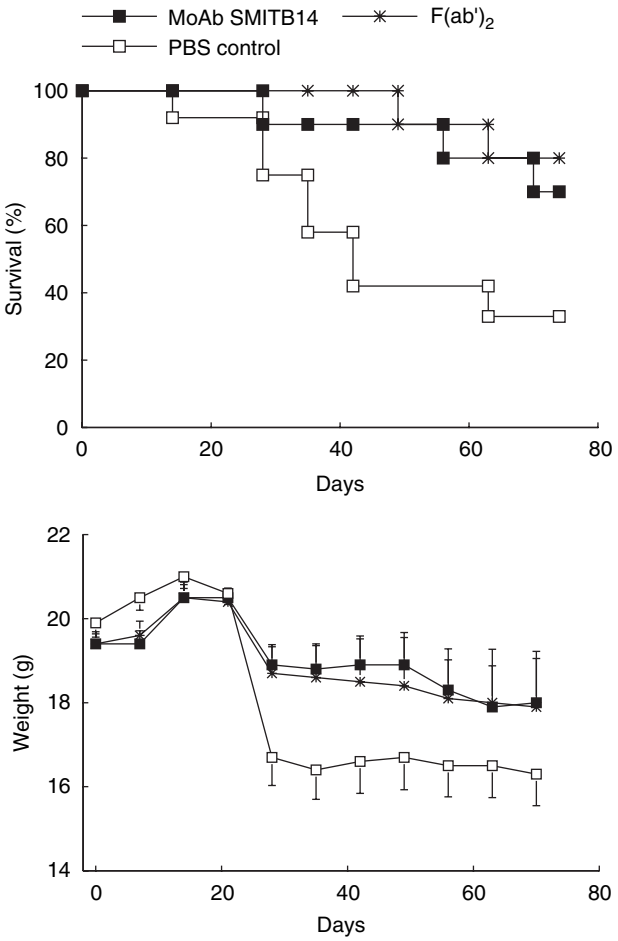

Fig. 4. Survival (upper panel) and body weight (lower panel) of BALB/c mice infected intravenously with 10^5 CFU *M. tuberculosis* Harlingen alone or in combination with MoAb SMITB14 or its F(ab′)$_2$ fragment (0·25 mg per mouse). For details see Materials and methods. (n = 10–12 mice per group).

were challenged i.v. with *M. tuberculosis* Harlingen alone, or *M. tuberculosis* Harlingen in combination with either MoAb SMITB14 or its F(ab′)$_2$ fragment. The mice were monitored for 70 days, their individual body weights were determined on the day of infection and then weekly after infection (Fig. 4). Over the entire observation period there was less weight loss and reduced mortality in groups of mice challenged with bacteria together with MoAb or F(ab′)$_2$ fragment compared to mice challenged with bacteria alone ($P = 0{\cdot}034$ and $P = 0{\cdot}008$ by log rank test, respectively). At the end of the observation period, seven of 10 mice in the MoAb group and 8/10 mice in the F(ab′)$_2$ group had survived, compared to 4/12 mice in the control group.

In another experiment (data not shown) mice were given i.v. *M. tuberculosis* Harlingen together with MoAb SMITB14 or its F(ab′)$_2$ fragment while control groups of mice received respective amounts of isotype control MoAb (SMIHHG1). In this experiment the survival was monitored over an extended period of 180 days. The mice challenged with *M. tuberculosis* in the presence of either MoAb SMITB14 or its F(ab′)$_2$ fragment survived significantly longer than their counterparts that received irrelevant MoAb ($P = 0{\cdot}017$ and $P = 0{\cdot}0003$, respectively). At the end of the experiment, three of 10 mice still remained alive and apparently healthy in the group given MoAb SMITB14 while all mice that received isotype control MoAb were dead.

The next experiment was performed in order to determine whether systemic administration of MoAb SMITB14 prior to infection would protect mice from weight loss and death (Fig. 5). Mice were inoculated i.v. with MoAb SMITB14, followed 60 min later by i.v. challenge with *M. tuberculosis* Harlingen. Control mice received isotype control MoAb SMIHHG1 prior to challenge infection. Also in this experimental setting mice were protected in terms both of prolonged survival (Fig. 5a, $P = 0{\cdot}013$) and reduced weight loss (Fig. 5b) over the study period of 190 days.

Effect of MoAb SMITB14 on bacterial burden in lungs of intranasally infected mice

One of the major questions regarding antimycobacterial immunity is whether relevant immune mechanisms, including antibodies, operate at the site of infection i.e. on the surface of the lung mucosa. In order to address this question an experiment was performed where F(ab′)$_2$ fragments from MoAb SMITB14 and *M. tuberculosis* Harlingen were administered simultaneously i.n. to BALB/c mice. Mice were sacrificed 6, 24 and 72 h later, lungs were isolated and bacterial loads were determined (Fig. 6). Early after infection (6 h) bacterial counts in lungs from mice that received *M. tuberculosis* together with F(ab′)$_2$ fragment were similar to those found in mice infected with *M. tuberculosis* alone. At 24 h, however, there was considerable decrease in bacterial loads in lungs of mice from the F(ab′)$_2$ group relative to the control group. The reduction in number of bacteria further progressed and at 72 h the difference between the F(ab′)$_2$ group and the control group became significant ($P < 0{\cdot}01$).

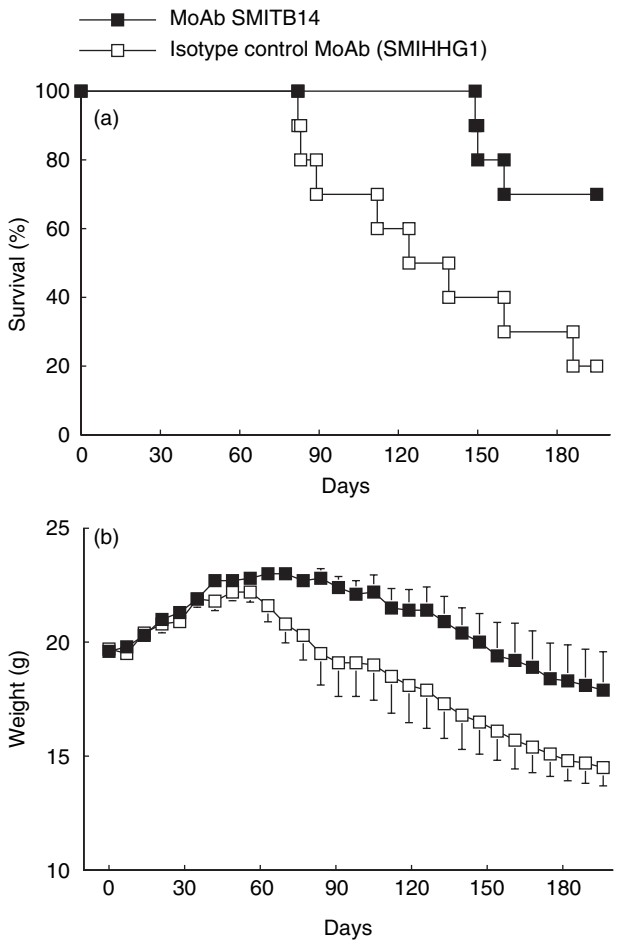

Fig. 5. Survival (a) and body weights (b) of BALB/c mice infected intravenously with 10^5 CFU *M. tuberculosis* Harlingen given 60 min after passive transfer of 0·1 mg of MoAb SMITB14. MoAb SMIHHG1 directed against human haemoglobin was used as isotype control antibody. ($n = 10$ mice per group).

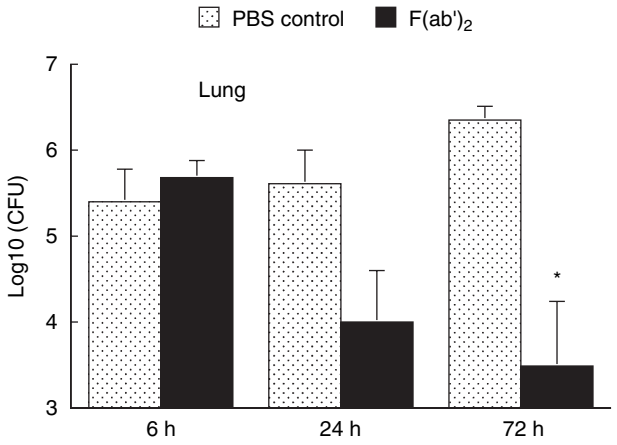

Fig. 6. Recovery of mycobacteria from lungs of BALB/c mice at different times after intranasal infection with 10^5 CFU *M. tuberculosis* Harlingen alone, or in combination with, 0·5 mg of $F(ab')_2$ fragment from MoAb SMITB14. Mean $\log_{10}$ (CFU) ± SEM from nine mice per group are shown. Asterisk indicates statistically significant difference between control and $F(ab')_2$ group ($P < 0.003$).

DISCUSSION

In tuberculosis, the current consensus is that only the cellular arm of the immune system is of importance for the effective control of the infection/disease. However, as in all infections, and also in natural *M. tuberculosis* infection and disease, the immune response of the host involves both the cellular and humoral immunological arms. Nevertheless, the possible beneficial or detrimental effects of elicited antibodies have largely been ignored. Therefore, in this study we have carried out a number of passive protection experiments in the *M. tuberculosis* infection model in mice to evaluate the possible beneficial/adverse effects mediated by a MoAb (SMITB14) with specificity for the polysaccharide portion of LAM, which is one of the major surface antigens of tubercle bacteria. In this model we found that the MoAb SMITB14 and its $F(ab')_2$ fragment significantly protected BALB/c mice against experimental *M. tuberculosis* infection. This protection was evidenced by a reduction in CFU counts in spleen and lung, by inhibition of the weight loss and, most importantly, by prolonged long-term survival (Figs 2–6). A control MoAb (specific for human haemoglobin) of the same IgG1 subclass and its $F(ab')_2$ fragment did not protect against *M. tuberculosis* challenge (Figs 2 and 5).

Prominent clinical features of TB in humans are fever and weight loss (wasting). In the mouse model, wasting is, in our experience, a good marker for the final outcome of acute TB. In particular, a negative weight development at the critical time period 2–4 weeks after challenge usually indicates that the mice will succumb early to the infection. Fever and weight loss have been attributed, at least in part, to the increased production of proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin (IL)-1α and IL-1β. These cytokines have been shown previously to be induced by stimulation of mononuclear cells with LAM [11–14]. Because the mice given MoAb SMITB14 or the corresponding $F(ab')_2$ together with *M. tuberculosis* did not lose as much weight as the mice given the control MoAb it is tempting to speculate that the anti-AM MoAb may inhibit the induction of proinflammatory cytokines by either the blockage of a crucial domain of LAM on the bacterial surface or by scavenging the released LAM. Similar effects have been reported previously in terms of the ability of O-antigen specific antibody to down-regulate the fever response induced by O-antigen homologous endotoxin in rabbits [15].

LAM is also known to have other biological effects on several components of the innate immune system, which are realized through interactions with a wide variety of host cells. Hence, LAM isolated from both *M. tuberculosis* and *M. leprae* has been reported to suppress T cell proliferation [16–18] and to interfere with gamma interferon-mediated activation of macrophages [19,20]. Other reported effects include inhibition of protein kinases [19], inhibition of the synthesis of mRNA encoding IL-2, IL-5, and granulocyte-macrophage colony stimulating factor (GM-CSF) in human T cells [21] and activation of the complement cascade [22]. MoAb SMITB14 may interfere with one or more of these potentially pathogenic effects of LAM, and this could conceivably contribute to the protection seen with this antibody.

The possibility that, in the presence of MoAb SMITB14, bacterial aggregates were formed which, by virtue of their size, would have their infectious potential diminished seems unlikely

because: (1) plating of a mixture of *M. tuberculosis*-specific MoAb yielded CFU counts identical to those obtained with *M. tuberculosis* alone or *M. tuberculosis*-isotype control MoAb mixture; (2) specific MoAb passively transferred, prior to *M. tuberculosis* challenge, was as protective as MoAb preincubated with bacteria; and (3) the amounts of bacteria recovered very early (6 h) from lungs of intranasally infected mice were similar, irrespective of the presence of MoAb.

Structurally, LAM consists of a mannan polysaccharide backbone core, substituted with terminal oligoarabinosyl side chains. The mannan core polysaccharide backbone is in turn attached to a phosphatidyl inositol lipid moiety [23,24]. LAM is a biologically polymorphic structure, and two major variants have been reported; one with the arabinomannan core substituted by arabinofuranosyl containing termini (AraLAM) and another where these termini are mannose-capped (ManLAM). The LAM used for generation of MoAb SMITB14 was prepared from *M. tuberculosis* H37Rv and was, by gel electrophoresis and Western blot using anti-Erdman LAM MoAbs, identical to the LAM prepared from *M. tuberculosis* Erdman [8], both strains reported to be heavily mannosylated. Western blot of MoAb SMITB14 against LAM showed abolition of binding after $NaIO_4$ treatment of LAM. In ELISA, the binding of MoAb SMITB14 to LAM was inhibited by AM in a dose-dependent fashion, showing that MoAb SMITB14 binds to the AM portion of LAM. MoAb SMITB14 also bound to the AM oligosaccharides conjugated to various types of proteins [25], demonstrating further the specificity of MoAb SMITB14 for the AM portion of LAM. The binding was not inhibited by yeast mannan, methyl α-D-mannopyranosides or DL-arabinose, indicating that the specificity of MoAb SMITB14 is directed to epitopes located outside of the mannose-capped region of LAM.

Antibodies could participate in protective immunity by many different mechanisms. The finding that MoAb SMITB14 binds to whole bacteria indicated that this antibody might be involved in blocking of a cellular invasion. This was corroborated by the findings that both i.v. and i.n. administration of this MoAb resulted in very early reduction of bacterial loads.

The fate of tubercle bacilli within a host phagocyte depends on the pathway by which the bacteria reach the intracellular milieu of the phagocytic cell. Different macrophage opsonic and non-opsonic receptors have been reported to mediate this uptake [26,27]. It has been suggested that receptors specific for surface carbohydrate epitopes may allow mycobacteria to bypass the bactericidal activity of macrophages [28]. LAM is known to mediate *M. tuberculosis* uptake by phagocytic cells, such as macrophages and dendritic cells and has been reported to utilize at least two receptors, CD14 and the murine mannose receptor, for entry into host phagocytes [29,30]. MoAb SMITB14 may prevent the uptake of *M. tuberculosis* through receptor(s) that facilitate bacterial survival and growth. It could be hypothesized that the MoAb SMITB14 binding to the surface of the invading tubercle bacilli leads to their uptake by particular cell populations that are specially effective in either direct killing or mediating proper cytokine/cellular signals to other effector cells with killing abilities.

Mice given MoAb SMITB14 lived significantly longer than control mice. Also, mice given $F(ab')_2$ fragment of MoAb SMITB14 survived longer than mice given control $F(ab')_2$ fragment. Protection afforded by the $F(ab')_2$ fragments indicates lack of involvement of the Fc phagocytic receptor [31]. It can also be speculated that anti-LAM antibodies and their $F(ab')_2$ fragments, by binding to LAM, could interfere with LAM-mediated inhibition of phagosome-lysosome fusion [32]. Recently, Teitelbaum *et al*. reported that a murine MoAb of IgG3 subclass (MoAb 9d8), also reactive with arabinomannan, partially protected C57BL/6 and BALB/c mice from death after respiratory challenge with a clinical isolate of Mtb [33]. In the same study, another MoAb of IgM subclass with LAM specificity did not affect the course of infection in mice. The protective IgG3 MoAb used by Teitelbaum *et al*. did not reduce the bacillary load in the lungs of infected animals, but it changed the distribution of the mycobacteria within the lung granulomas. In our studies we did not examine the distribution of the bacteria in lungs but in contrast to these investigators we noticed a significant reduction in bacterial load in the lungs. This latter finding might, at least, in part be due to our use of the i.v. challenge route, while Teitelbaum *et al*. [33] used the intratracheal route.

Studies of *M. tuberculosis* infections in gene-deleted mice, incapable of making B cells, are often regarded as providing definitive evidence of the lack of role of antibodies in antimycobacterial defence. However, the results of three studies published so far on *M. tuberculosis* infections in B cell-deficient mice seem contradictory and do not allow for the dismissal of a role for antibody. Vordermeier *et al*. reported that i.v. challenge resulted in significantly higher bacterial counts in organs of B cell-deficient mice, compared to the wild strain, but no difference in survival ensued [34], Johnson *et al*. reported no difference in bacterial loads or early lung pathology upon aerosol challenge (survival was not recorded) [35], while Bosio *et al*. reported that aerosol challenge resulted in similar lung bacterial loads but considerably different pathology in B cell-deficient and wild-type mice [36]. Furthermore, the observation by Bosio *et al*. [36], that the supplementation of the deficient mice with passively transferred whole immune serum did not lead to the reversal of observed changes, should be treated with caution. It is conceivable that the presence of the whole repertoire of antibodies, such as those present during 'natural' immune response [37] or in passively transferred whole serum, may result in neutralization of the effect of beneficial antibodies by disadvantageous ones. Thus, it has been shown in other systems, that the fine specificity and isotype [33,38], and the amount of an individual MoAb [39] may decide whether or not it is advantageous for the host. In addition, a paradoxical loss of efficacy for certain antibodies, passively transferred at high doses, has been described [39]. Considering the variety of polyclonal antibodies raised, in response to a multitude of mycobacterial antigens in the course of infection, it may be argued that antibodies of a particular isotype against a specific antigen/epitope could be of clinical interest, e.g. in preparing a new effective vaccine against TB.

To what extent the humoral immune response plays a role in innate and acquired immunity to active TB in man still remains an enigma. There has been no extensive research on the potential association between antibody deficiency and the risk of acquiring either infection or active TB, although sporadic cases have been reported. Accordingly, a somewhat higher incidence of TB has been reported in patients with hypogammaglobulinaemia [40].

The findings presented here that passively transferred antibodies against AM resulted in significant protection against TB have encouraged us to study the possible protective effects of LAM oligosaccharide-based protein conjugates as putative vaccine candidates. Indeed, these conjugate vaccines – when administered in proper adjuvants – proved to elicit not only cell-mediated

immune responses but also high anti-LAM IgG antibody titres [8] and protected animals efficiently against TB [25]. We suggest that the antibodies elicited by these new vaccine candidates, at least in part, mediated this protection.

In summary, the observations reported in the present study indicate further that certain antibodies may be protective in experimental TB and justify the need for continued studies to clarify the role of humoral responses in the natural course of TB as well as in vaccine-induced immunity.

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