INFECTION AND IMMUNITY, May 1997, p. 1856–1862 Vol. 65, No. 5
0019-9567/97/$04.00+0

Induction of a Type 1 Immune Response to a Recombinant Antigen from *Mycobacterium tuberculosis* Expressed in *Mycobacterium vaccae*

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Received 26 November 1996/Returned for modification 2 January 1997/Accepted 20 February 1997

A 19-kDa lipoprotein from *Mycobacterium tuberculosis* was expressed as a recombinant antigen in the nonpathogenic mycobacterial host strain *M. vaccae*. Immunization of mice with the recombinant *M. vaccae* resulted in induction of a strong type 1 immune response to the 19-kDa antigen, characterized by immunoglobulin G2a (IgG2a) antibodies and gamma interferon (IFN-γ) production by splenocytes. Immunization with the same antigen in incomplete Freund's adjuvant induced a strong IgG1 response with only low levels of IFN-γ. Subsequent intravenous and aerosol challenges of immunized mice with virulent *M. tuberculosis* demonstrated no evidence of protection associated with the response to the 19-kDa antigen; in fact, the presence of the recombinant 19-kDa antigen abrogated the limited protection conferred by *M. vaccae* (vector control). The recombinant *M. vaccae* system is a convenient approach to induction of type 1 responses to *M. tuberculosis* antigens. However, the unexpected reduction in protective efficacy of *M. vaccae* expressing the 19-kDa antigen highlights the complexity of testing recombinant subunit vaccines and the need for a better understanding of the immune mechanisms required for effective vaccination against tuberculosis.

T-cell-mediated immune responses can be broadly categorized as type 1 or 2, based on the predominant cytokine profile expressed by the activated lymphocytes (21). A type 1 response is dominated by the production of gamma interferon (IFN-γ), which triggers activation of macrophages, enhancing their microbicidal functions. A type 2 response is characterized by synthesis of interleukin-4 (IL-4) and IL-5, which stimulate immunoglobulin E (IgE)-dependent mast cell degranulation and eosinophil activation. At the antibody level, the type 2 response in mice is associated with production of the IgG1 and IgE subclasses and the type 1 response in mice is associated with production of the IgG2a subclass (9). Protection against infection with intracellular pathogens requires macrophage activation mediated by a type 1 response (6, 10, 20). We are interested in exploring strategies for the induction of type 1 immune responses to antigens from *Mycobacterium tuberculosis*, with the eventual goal of developing subunit vaccines for the control of tuberculosis. In the present study, we have explored the use of a rapid-growing nonpathogenic mycobacterium, *M. vaccae*, as both an expression system and as an adjuvant for induction of responses to a 19-kDa antigen from *M. tuberculosis.*

The 19-kDa protein was originally identified as a major antigen of *M. tuberculosis* by using murine monoclonal antibodies (7) and was subsequently shown to be the target of both antibody and T-cell responses in the course of tuberculosis infection and *Mycobacterium bovis* BCG vaccination in humans (8, 13, 17). It is expressed predominantly in the form of a cell-associated lipoprotein (32) and is further modified by glycosylation (12). We have previously shown that posttranslational modification of the 19-kDa protein is reproduced when the *M. tuberculosis* gene is expressed in a rapid-growing mycobacterium, *M. smegmatis* (12). Related proteins are found in other slow-growing mycobacterial species (5, 22), but the physiological function of the protein is unknown.

M. vaccae is a soil organism which has been used in clinical trials as a potential immunotherapeutic agent for treatment of tuberculosis and other immune system-mediated diseases (29). In contrast to the BCG vaccine, *M. vaccae* is used in humans as a heat-killed preparation. The mechanisms underlying its possible therapeutic action remain to be clarified, but when administered at an appropriate dose, it has been shown to elicit a strong type 1 immune response (14). *M. vaccae* has been found to confer both prophylactic and therapeutic benefit against challenge with *M. tuberculosis* in a murine intratracheal infection model (26) but shows no significant protection in an immunotherapy model based on low-dose aerosol infection (2a). We reasoned that *M. vaccae* might provide a useful adjuvant system for induction of type 1 responses to added antigens. The availability of genetic systems for expression of recombinant antigens in *M. vaccae* provided an additional advantage (11), particularly in relation to *M. tuberculosis* antigens for which posttranslational modification may influence immunogenicity. We report on the immunogenicity of the *M. vaccae*-expressed 19-kDa antigen and the evaluation of protective efficacy against *M. tuberculosis* challenge.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *M. vaccae* NCTC11659 was supplied by John Stanford (University College London Medical School, London, United Kingdom). *M. vaccae* was grown at 30°C on Middlebrook 7H11 (Difco) agar plates or with shaking in 7H9 medium (Difco) supplemented with 2% glucose. Hygromycin B (Boehringer Mannheim) was added at 50 μg/ml for cultures of strains transformed with shuttle plasmids. *Escherichia coli* was grown on Luria-Bertani medium with the addition of ampicillin (100 μg/ml) and kanamycin (25 μg/ml) or hygromycin B (200 μg/ml) as appropriate. Plasmid p16R1-19 is a derivative of p16R1, a *Mycobacterium-E. coli* shuttle vector (11), containing a 1.8-kb *Sma*I fragment which includes the gene encoding the 19-kDa antigen of *M. tuberculosis* (4). The *Sma*I fragment was isolated from pRL19k2.8 (12) and inserted into the unique *Kpn*I site of p16R1. For the production of a nonacylated form of the 19-kDa antigen, a 400-bp DNA fragment encoding the mature protein without the lipoprotein leader sequence was cloned into the *Sma*I site of the vector pQE-30 (QIAGEN) to generate pQE-3019.

DNA manipulations. Standard procedures were used for transformation of *E. coli*, preparation of plasmids, and analysis of DNA by restriction enzyme digestion and agarose gel electrophoresis (27). Transformation of *M. vaccae* with shuttle plasmids was carried out by electroporation as described previously (11, 33).

Gel electrophoresis and Western blotting. For analysis of protein expression in transformants, bacterial extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous buffer system on slab gels of 15% acrylamide (18). Two-dimensional PAGE was performed on the purified *M. vaccae* recombinant 19-kDa protein by applying isoelectric focusing to tube gels containing 4% ampholytes in the pH range 4 to 6 and 1% ampholytes pH 3 to 10 (Serva) followed by SDS-PAGE in the second dimension. Gels were then stained with silver nitrate (19); in some gels, a periodic acid oxidation step (30) was applied following fixation to enhance the appearance of glycoproteins and lipoglycans.

For Western blot analysis, proteins were transferred from polyacrylamide gels onto nitrocellulose membranes (Hybond C; Amersham). Blots were then stained by using monoclonal antibodies specific for the *M. tuberculosis* 19-kDa antigen (7), HYT6 and F29-47, as described previously (1). Processing of nitrocellulose blots with peroxidase-conjugated concanavalin A (ConA) (Sigma) at a concentration of 5 μg/ml was similar to that used for antibody staining.

Preparation of antigens. For use in immunization experiments, recombinant *M. vaccae* was grown for 5 days at 30°C on 7H11 agar plates containing 2% glucose and 50 μg of hygromycin B per ml. Organisms were suspended in phosphate-buffered saline (PBS) containing 0.005% Tween 80 and heat-killed at 80°C for 20 min. Bacteria were washed twice in the same buffer, and aliquots of 10 mg (wet weight)/ml were stored at −20°C. Protein extracts from *M. vaccae* were prepared by sonicating bacterial suspensions in PBS; soluble antigens were separated from cell debris by centrifugation at 13,000 × *g* for 20 min and passed through 0.22-μm-pore-size filters (Millipore).

The 19-kDa antigen was purified from recombinant *M. vaccae* cells harvested after 6 days of culture. Bacteria were suspended in PBS–1 mM EDTA with protease inhibitors and disrupted by shaking with 0.1-mm-diameter glass beads in a bead beater (Stratech) for 15 30-s periods on ice. After centrifugation at 27,000 × *g* for 30 min to remove unbroken cells and cell walls, the resulting supernatant was fractionated by ammonium sulfate. The 19-kDa protein was precipitated with 25% ammonium sulfate and resuspended in 20 mM Tris-HCl (pH 8.4) with 10 mM *N*,*N*-bis-(3-D-gluconamidopropyl)deoxycholamide (deoxy-BIGCHAP; Boehringer Mannheim), a nonionic detergent. The protein was purified by anion-exchange and size exclusion chromatography, and residual detergent was removed by adsorption onto Extracti-Gel (Pierce). The nonacylated form of the 19-kDa protein, containing a six-His tag at the lysine residue at position 5 of the mature protein, was purified by affinity chromatography on nickel-nitrilo-tri-acetic acid resin from induced cultures of *E. coli* transformed with pQE-3019, as described in the QIAGEN protocol. The total protein concentration was estimated by the bicinchoninic acid protein assay (Pierce) with bovine serum albumin (BSA) as the standard.

Immunization protocols. Female C57BL/6 mice (Harlan, Bicester, United Kingdom), aged 6 to 8 weeks, in groups of three or four, were injected subcutaneously in the flanks with the appropriate dose of antigen in 0.2 ml and given booster injections 3 weeks later with the same immunogen. Mice received 0.1 mg (corresponding to 10^8 organisms) of *M. vaccae* expressing the 19-kDa antigen, 0.1 mg of *M. vaccae* (vector control) mixed with 0.2 μg of purified recombinant 19-kDa protein, or 19-kDa protein (0.2, 10, or 50 μg) emulsified in incomplete Freund's adjuvant (IFA). Control animals were immunized with 0.1 mg of *M. vaccae* (vector control) or with PBS emulsified in IFA. On day 31, the mice were bled, their spleens were removed, and single-cell suspensions were prepared and cultured with antigens as described below.

Antibody analysis by enzyme-linked immunosorbent assay (ELISA). Microtiter plates (Maxisorp-Immuno plates; Nunc) were coated overnight at 4°C with an optimal concentration of the different antigens in 0.1 M carbonate buffer, pH 9.6. The recombinant 19-kDa antigen and *M. vaccae* extract were coated at 1 and 5 μg/ml, respectively. Plates were blocked for 1 h at 37°C with 1% BSA in 0.05 M Tris-HCl-buffered 0.15 M saline (pH 8.0) (TBS). Plates were washed with TBS containing 0.05% Tween 20 (TBST) and incubated with serum samples diluted in blocking solution for 2 h at 37°C. After three washes with TBST, the plates were probed for 2 h at 37°C with alkaline phosphatase-conjugated rat anti-mouse IgG1 or IgG2a monoclonal antibody (Pharmingen) diluted 1:1,000 and 1:1,500, respectively. After a further three washes, the plates were incubated with phosphatase substrate (*p*-nitrophenylphosphate [1 mg/ml] in 0.2 M Tris buffer [pH 9.8]; Sigma). The absorbance was read at 405 nm (microplate reader 3550; Bio-Rad). In experiments to monitor the specificity of secondary antibody reagents, doubling dilutions (2 μg/ml to 19 ng/ml) of purified mouse IgG1 and IgG2a myeloma proteins (Serotec) were similarly coated onto wells; anti-IgG1 and anti-IgG2a antibodies showed no detectable cross-reactivity under these conditions with IgG2a and IgG1, respectively.

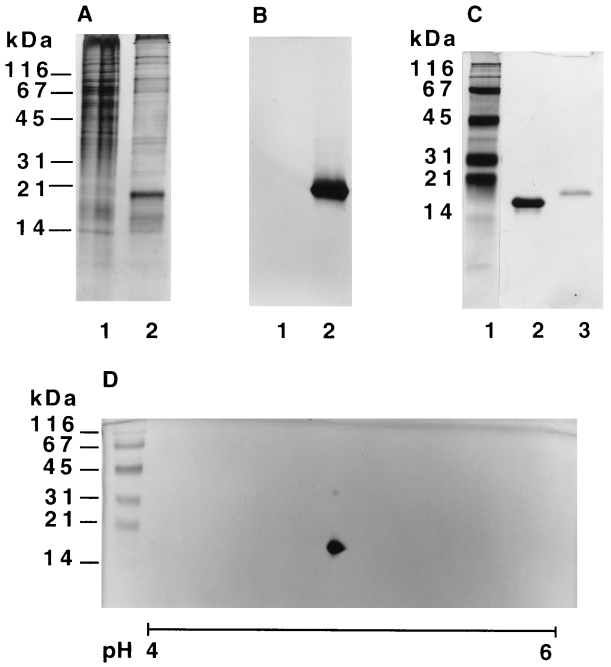

FIG. 1. Expression of the *M. tuberculosis* 19-kDa antigen in *M. vaccae* and analysis of purified recombinant proteins. Mycobacterial extracts were analyzed by SDS-PAGE and stained for total protein with silver nitrate (A) or by immunoblot analysis with monoclonal antibody HYT6 (B). Migration positions of molecular mass markers are shown on the left. Lane 1, *M. vaccae* (vector control); lane 2, *M. vaccae* expressing the 19-kDa antigen. (C) Analysis of the recombinant 19-kDa antigens purified from *E. coli* (lane 2) and *M. vaccae* (lane 3) by SDS-PAGE and silver staining. Lane 1, molecular mass markers. (D) Analysis of the purified *M. vaccae* recombinant protein by two-dimensional PAGE and silver staining.

In vitro assay for cytokine production by spleen cells. Spleens were removed aseptically from mice and placed in RPMI 1640 medium (Gibco BRL) supplemented with 5% fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml). Single-cell suspensions were prepared by crushing the spleens through a cell strainer (100-μm nylon mesh [Falcon 2360; Becton Dickinson]). Splenocytes were washed, counted by using 0.01% crystal violet (in 0.01% acetic acid in distilled water), and plated at 8×10^5 cells/well in 96-well flat-bottomed plates. Cells were stimulated with the recombinant 19-kDa antigen purified from *E. coli* or *M. vaccae* (2 and 10 μg/ml), *M. vaccae* extract (5 μg/ml), and ConA (2.5 μg/ml). Control wells contained medium alone. Supernatants were collected at 18, 72, and 96 h for the IL-4 assay, at 48 and 72 h for the IFN-γ assay, and at 72 and 96 h for the IL-5 assay.

Cytokine assays. Supernatants harvested from splenocyte cultures were screened by ELISA or by bioassay using standard procedures. IFN-γ and IL-5 were assayed by ELISA with antibody pairs purchased from Pharmingen as follows: for IFN-γ, clones R4-6A2 and XMG1.2 (sensitivity, 312 pg/ml); for IL-5, clones TRFK5 and TRFK4 (sensitivity, 24 pg/ml). Biotinylated detecting antibodies were used in conjunction with streptavidin-alkaline phosphatase conjugate (Amersham). Plates were developed with phosphatase substrate as described above. The concentrations of IFN-γ were calculated from a standard curve for recombinant mouse IFN-γ (Pharmingen) generated in each assay. IL-4 levels were measured by using [^{3}H]thymidine uptake of the IL-4-dependent CT4S cell line and comparing it with dilutions of recombinant IL-4.

Protection studies. We used two models to test the effects of immunization with the *M. vaccae* recombinant on susceptibility of mice to subsequent challenge with *M. tuberculosis.*

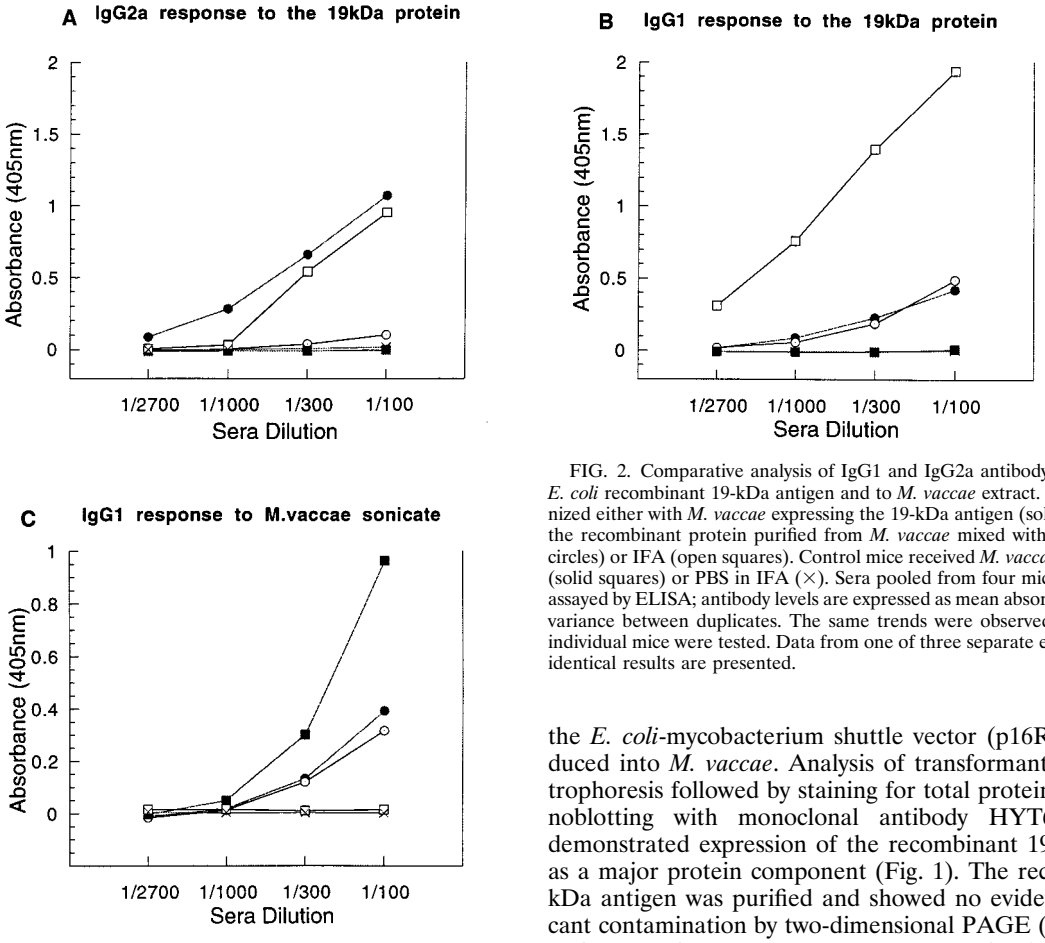

FIG. 2. Comparative analysis of IgG1 and IgG2a antibody responses to the *E. coli* recombinant 19-kDa antigen and to *M. vaccae* extract. Mice were immunized either with *M. vaccae* expressing the 19-kDa antigen (solid circles) or with the recombinant protein purified from *M. vaccae* mixed with *M. vaccae* (open circles) or IFA (open squares). Control mice received *M. vaccae* (vector control) (solid squares) or PBS in IFA (×). Sera pooled from four mice per group were assayed by ELISA; antibody levels are expressed as mean absorbance with <10% variance between duplicates. The same trends were observed when sera from individual mice were tested. Data from one of three separate experiments giving identical results are presented.

(i) Lethal challenge model. C57BL/6 mice (bred at the Central Institute for Tuberculosis, Moscow, Russia) in groups of 25 were immunized subcutaneously in the dorsum either with 10^8 *M. bovis* BCG (Prague substrain), *M. vaccae* expressing the 19-kDa antigen, or *M. vaccae* (vector control) in 0.5 ml of PBS, or with 10 μg of purified recombinant antigen emulsified in IFA and then given a booster injection with the same dose of each antigen 3 weeks later. Control mice received PBS in IFA. Sera were collected from five mice from each group 10 days after the booster injections. Three weeks following vaccination, mice were challenged by intravenous injection with 5×10^5 *M. tuberculosis* H37Rv (23). *M. tuberculosis* H37Rv was prepared from 3-week-old cultures on Loewenstein-Jensen medium and stored at −70°C in sterile saline, containing 0.05% Tween 80 and 0.1% BSA until used. Three mice from each group were sacrificed at weekly intervals; bacterial colony formation was determined by plating serial dilutions of spleen homogenates on Dubos agar. Resistance was measured also as the mean survival time (MST) (3) for 8 to 12 mice in each group.

(ii) Aerosol challenge model. Female C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine) were injected subcutaneously with 10^6 *M. vaccae* (vector control) or *M. vaccae* expressing the 19-kDa antigen in 0.2 ml of saline. Positive-control mice were injected with 10^6 *M. bovis* BCG (Pasteur substrain); for negative controls, mice were given an injection of saline. Four weeks following inoculation, mice were exposed to a challenge infection in which approximately 50 *M. tuberculosis* Erdman bacilli were delivered into the lungs with an aerosol generation device (Glas-Col, Terre Haute, Indiana). Thirty days later, numbers of bacteria in the lungs of these mice were determined by plating serial dilutions of individual whole-organ homogenates on nutrient Middlebrook 7H11 agar, and colonies were counted after 3 to 4 weeks of incubation.

Statistical analysis. Data analysis was performed by using Student's *t* test. Differences between the mouse groups were considered significant if *P* values were <0.05.

RESULTS

Expression of the 19-kDa lipoprotein in *M. vaccae*. The *M. tuberculosis* gene encoding the 19-kDa antigen was cloned in the *E. coli*-mycobacterium shuttle vector (p16R1) and introduced into *M. vaccae*. Analysis of transformants by gel electrophoresis followed by staining for total protein or by immunoblotting with monoclonal antibody HYT6 or F29-47 demonstrated expression of the recombinant 19-kDa antigen as a major protein component (Fig. 1). The recombinant 19-kDa antigen was purified and showed no evidence of significant contamination by two-dimensional PAGE (Fig. 1D). The 19-kDa product gave one strong spot and a faint spot (with identical pI) and with molecular weights of approximately 19,000 and 37,000, and both spots reacted with monoclonal antibody F29-47. The purified recombinant protein was used to standardize a semiquantitative SDS-PAGE assay. Based on this assay, the level of expression of the 19-kDa antigen was estimated as 0.2 μg of protein per 0.1 mg (wet weight) of bacterial pellet (corresponding to 10^8 bacteria). As previously described for a similar construct in *M. smegmatis*, the recombinant antigen was found predominantly as a cell-associated lipoprotein, with ConA-binding properties indicative of glycosylation (data not shown). The purified recombinant protein is currently undergoing structural analysis for full characterization of acyl and carbohydrate constituents. Preliminary results indicate covalent association of both mannose and *N*-acetylhexosamine residues (30a).

The 19-kDa antigen was also expressed in *E. coli* (Fig. 1). By using the QIAexpress pQE vector, a histidine tag was incorporated at the N terminus of the mature protein to generate a nonacylated form of the protein. The purified *E. coli* recombinant protein did not bind ConA (data not shown).

Antibody responses to the 19-kDa antigen. Three different approaches were used to immunize C57BL/6 mice with the 19-kDa antigen. Groups of mice were immunized either with *M. vaccae* expressing the 19-kDa antigen or with the recombinant protein purified from *M. vaccae* mixed with *M. vaccae* or IFA. Control mice received IFA or *M. vaccae* (vector control). A booster injection was given 21 days after the initial immunization, and immune responses were analyzed on day 31. Figure 2 illustrates titration of IgG1 and IgG2a antibodies in

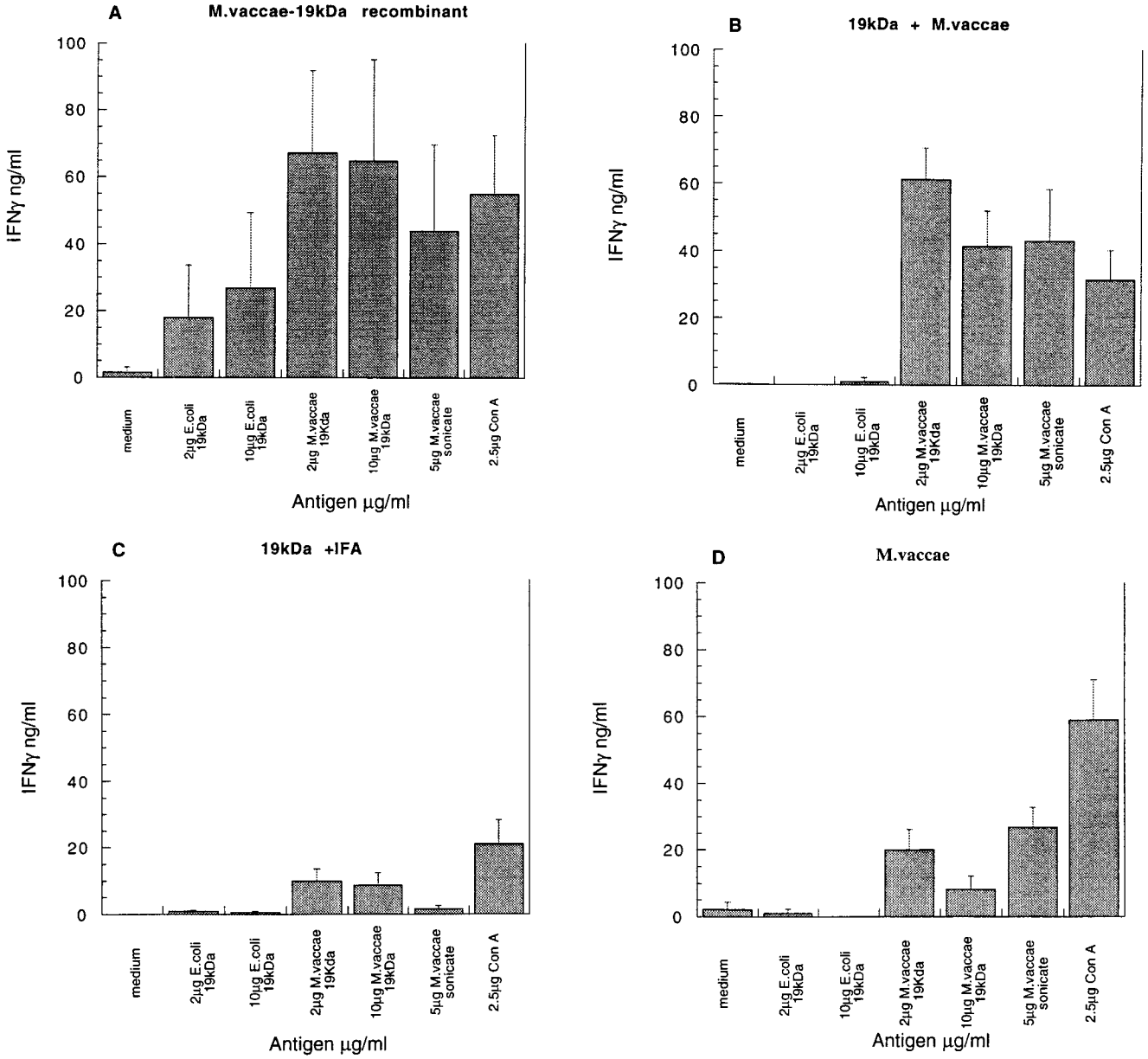

FIG. 3. Antigen-stimulated IFN-γ secretion in vitro by splenocytes following immunization. Mice were immunized either with *M. vaccae* expressing the 19-kDa antigen (A), with the recombinant protein purified from *M. vaccae* mixed with *M. vaccae* (B) or IFA (C), or with *M. vaccae* (vector control) (D). Each bar represents the mean concentration in 48-h culture supernatants for the entire group ± standard deviation. Results from one representative experiment of three are shown.

sera from different groups, using the nonacylated *E. coli* recombinant protein or *M. vaccae* extract as the antigen. Immunization with *M. vaccae* expressing the 19-kDa antigen induced a strong IgG2a response (Fig. 2A) and a weak IgG1 response (Fig. 2B) to the 19-kDa protein. In contrast, immunization with the purified recombinant protein in IFA generated strong IgG2a (Fig. 2A) and IgG1 (Fig. 2B) responses to the 19-kDa antigen across a wide range of immunizing antigen doses (0.2 to 50 μg of protein). The results shown in Fig. 2 are those obtained with an immunizing dose of 0.2 μg of protein, equivalent to that present in the recombinant clone. Immunization with the purified protein mixed with *M. vaccae* produced detectable, albeit low, IgG1 levels comparable to those induced by the *M. vaccae* expressing the 19-kDa antigen but negligible levels of IgG2a. Immunization with *M. vaccae* (vector control) resulted in no detectable antibody response to the 19-kDa antigen, suggesting the absence of a cross-reacting endogenous homolog of the 19-kDa protein. The recombinant 19-kDa protein purified from *M. vaccae* generated ELISA results similar to those obtained with the *E. coli*-derived protein (data not shown), suggesting that the antibody response is directed predominantly to protein, rather than acyl or carbohydrate, determinants. All animals immunized with *M. vaccae*-containing preparations produced antibodies to *M. vaccae* sonicate; total IgG1 antibody levels were higher in mice that received *M. vaccae* (vector control) than in mice that received *M. vaccae* expressing the 19-kDa antigen or *M. vaccae* mixed with this protein (Fig. 2C).

TABLE 1. Susceptibility to a lethal form of experimental tuberculosis following immunization with *M. vaccae* expressing the 19-kDa antigen, *M. vaccae* (vector control), or the purified recombinant 19-kDa antigen mixed with IFA

Immunization	Antibody isotype to 19-kDa antigen[a]		CFU in spleen[b]		
	IgG1	IgG2a	Week 1	Week 2	Week 3
M. vaccae expressing the 19-kDa antigen	0.81	7.97	$(9 \pm 1.6) \times 10^4$	$(5 \pm 0.6) \times 10^5$	$(1 \pm 0.2) \times 10^7$
M. vaccae			$(9 \pm 0.3) \times 10^4$	$(3 \pm 0.3) \times 10^5$	$(1 \pm 0.2) \times 10^6$
19-kDa antigen in IFA	164.65	4.82	$(5 \pm 0.4) \times 10^5$	$(4 \pm 0.5) \times 10^5$	$(8 \pm 2.2) \times 10^6$
IFA (control)			$(3 \pm 0.1) \times 10^5$	$(1 \pm 0.2) \times 10^6$	$(1 \pm 0.2) \times 10^7$

[a] Serum samples from five mice per group were pooled and tested by ELISA. Levels of specific IgG subclasses against the 19-kDa antigen are expressed as mean concentrations in micrograms per milliliter.

[b] Mice challenged with *M. tuberculosis* were sacrificed at weekly intervals (three mice from each group). The number of live bacteria was assessed in spleens and is shown as a mean number of CFU ± standard deviation.

Splenocyte responses to the 19-kDa antigen. The T-cell response to the 19-kDa antigen was analyzed by measuring the release of IFN-γ by splenocytes cultured in the presence of purified 19-kDa protein from *E. coli* or *M. vaccae* (Fig. 3). IFN-γ production was similar at both time points, 48 and 72 h; the results for IFN-γ shown in Fig. 3 were determined in culture supernatants harvested after 48 h. Spleens from mice immunized with the *M. vaccae* expressing the 19-kDa antigen expressed the highest levels of IFN-γ (Fig. 3A). These mice responded to recombinant forms of the 19-kDa protein purified from *E. coli* and *M. vaccae*, with the latter inducing the strongest response. Spleens from mice immunized with the 19-kDa protein mixed with *M. vaccae* responded to the *M. vaccae*-expressed protein but showed little or no response to the *E. coli* recombinant (Fig. 3B). Mice immunized with the 19-kDa protein in IFA or with *M. vaccae* (vector control) showed weak responses only to the *M. vaccae*-expressed antigen (Fig. 3C and D). A sonicate of *M. vaccae* induced an IFN-γ response in all of the groups immunized with *M. vaccae*-containing preparations. Spleens from a control group of mice immunized with PBS in IFA released IFN-γ only in response to ConA stimulation (data not shown). We were unable to detect significant amounts of IL-4 (lower assay limit, 0.5 ng/ml) in supernatants from splenocyte cultures stimulated either with the recombinant 19-kDa antigen or with *M. vaccae* extract at early (18 h) or late (72 and 96 h) time points. Incubation with ConA consistently induced positive IL-4 responses (1.5 to 3 ng/ml) at 18 h. No IL-5 was produced under these experimental conditions (data not shown).

Induction of a strong IFN-γ response against the 19-kDa protein, together with the relative predominance of IgG2a antibodies, demonstrate that immunization with *M. vaccae* expressing the 19-kDa antigen elicits a type 1 immune response to this antigen. This finding encouraged us to test the effect of this immunization strategy on susceptibility of mice to challenge with *M. tuberculosis.*

Resistance to *M. tuberculosis*. (i) High-dose lethal challenge model. Groups of mice were immunized as described above with *M. vaccae* expressing the 19-kDa antigen or with the purified recombinant antigen mixed with IFA. Control groups received PBS in IFA or *M. vaccae* (vector control). The effect of immunization was monitored by serological tests and confirmed the inverse IgG1/IgG2a ratios conferred by different protocols (Table 1). Three weeks after the booster injection, mice were challenged with 5×10^5 *M. tuberculosis* H37Rv. CFU were determined in spleens at weekly intervals (Table 1), and the survival times for 8 to 12 mice from each group were recorded (Fig. 4). Immunization with *M. vaccae* (vector control) conferred some protection, as evidenced by a 10-fold reduction in the number of bacteria at week 3 (Table 1) and the 12-day increase in MST (34.4 days ± 2.0) (Fig. 4). This increase is relatively modest in comparison to that conferred by live BCG in this model (9 to 10 weeks) (3), but the MST differs significantly from that of the control group (22.8 days ± 0.63 [$P < 0.05$]). In contrast, animals immunized with *M. vaccae* expressing the 19-kDa antigen or with the purified 19-kDa antigen in IFA were indistinguishable from the control animals. Thus, despite the establishment of a type 1-like response to the 19-kDa antigen, immunization with *M. vaccae* expressing the 19-kDa antigen failed to confer protection against tuberculous challenge. In fact, expression of the 19-kDa recombinant in *M. vaccae* abrogated the weak protection induced by the bacterium alone.

(ii) Low-dose aerosol challenge model. The *M. vaccae* immunization protocol was also assessed in a second experimental system involving aerosol challenge and analysis of bacterial counts in the lungs (Table 2). Immunization with BCG in this model results in a 10-fold reduction in bacterial counts. Immunization with *M. vaccae* (vector control) resulted in a slight but statistically unsignificant reduction in mean bacterial counts, whereas immunization with *M. vaccae* expressing the 19-kDa antigen has led to an increase in the bacterial count over that of control mice, although this difference again was not significant. Thus, in both high-dose lethal challenge and

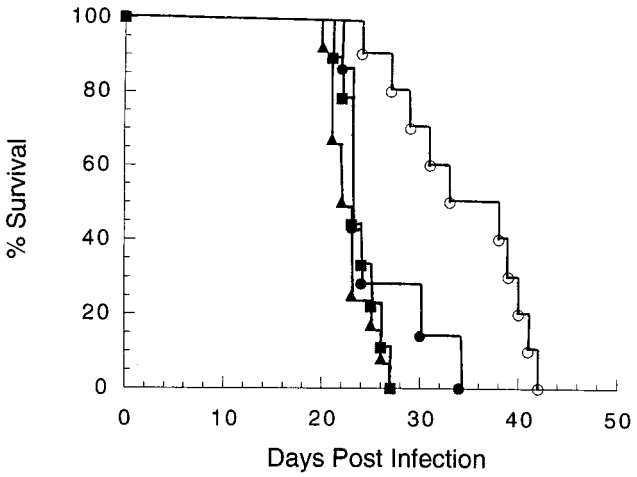

FIG. 4. Survival times of mice immunized with *M. vaccae* expressing the 19-kDa antigen (solid circles), *M. vaccae* (vector control) (open circles), the recombinant 19-kDa antigen in IFA (solid squares), or PBS in IFA (solid triangles) (negative control) and then challenged with 5×10^5 *M. tuberculosis*. Data are for 8 to 12 mice per group.

TABLE 2. Comparative efficacy of the *M. vaccae* immunization against aerosol challenge with *M. tuberculosis*[a]

Immunization	Log_{10} bacteria (CFU) in lungs
M. vaccae expressing the 19-kDa antigen	4.55 ± 0.40
M. vaccae	3.65 ± 0.47
BCG	3.16 ± 0.10
Saline	4.00 ± 0.11

[a] Mice were immunized 4 weeks prior to challenge with *M. tuberculosis*. The number of CFU in lungs was measured 30 days later.

low-dose aerosol challenge models, no protection was conferred by the response to the 19-kDa antigen.

DISCUSSION

M. vaccae provides a convenient host for expression of the *M. tuberculosis* 19-kDa glycosylated lipoprotein. Expression levels are approximately 10-fold higher than in *M. smegmatis*. As in the case of *M. smegmatis* (12), expression was obtained without addition of an exogenous promoter, and it is likely that *M. vaccae* is able to recognize natural *M. tuberculosis* expression signals. Similar results have been achieved with another *M. tuberculosis* protein—the superoxide dismutase enzyme—which is also expressed at higher levels in *M. vaccae* than in *M. smegmatis* (11, 33). Enhanced protein stability, perhaps associated with lower levels of proteolytic activity, may be an important factor providing these results (15).

Immunization with *M. vaccae* expressing the 19-kDa antigen induces a strong type 1-like immune response reflected by in vitro IFN-γ production by splenocytes and by IgG2a antibodies in sera of immunized mice. Immunization with the purified 19-kDa antigen in IFA also induces a strong antibody response of both the IgG1 and IgG2a isotypes and is associated with much lower levels of IFN-γ. Mixing the purified protein with *M. vaccae* leads to an intermediate response: a strong IFN-γ production but only low levels of IgG2a antibodies to the 19-kDa antigen.

The recombinant antigen purified from *M. vaccae* elicited a stronger IFN-γ response in splenocytes than the corresponding *E. coli*-derived recombinant protein. The *M. vaccae*-derived protein, in contrast to the *E. coli*-derived protein, is subject to posttranslational modifications, and it is possible that lipid or carbohydrate moieties influence its immune recognition. Alternatively, the protein purified from *M. vaccae* may contain some noncovalently associated contaminant capable of influencing the immune response. In support of this latter explanation, splenocytes from mice immunized with *M. vaccae* (vector control) responded to the 19-kDa protein purified from *M. vaccae*, while showing no response to the equivalent *E. coli* preparation (Fig. 3D). Immunization with the purified *M. vaccae*-expressed protein in IFA did not, however, induce any antibody or T-cell response to *M. vaccae* sonicate. Other researchers have similarly observed that antigens isolated from mycobacteria are superior to the corresponding *E. coli*-derived proteins in immunological assays (25, 28), and we are continuing to evaluate this phenomenon in separate experiments with altered forms of the 19-kDa protein generated by site-directed mutagenesis.

There is widespread recognition of the need for improved vaccines for control of tuberculosis (31), and several groups are searching for appropriate combinations of antigens and adjuvants for inclusion in new subunit vaccines. Antigens that are secreted from actively growing mycobacteria in culture medium currently are among the most promising candidates (2, 16, 24). Biochemical purification of proteins from *M. tuberculosis* cultures and their testing with different adjuvant preparations present major technical challenges; however, it is possible that use of recombinant expression strategies would facilitate screening of a range of individual antigens. The ability of *M. vaccae* to express *M. tuberculosis* proteins, together with its efficacy as an adjuvant inducing a preferential type 1 response, makes it an attractive system for antigen evaluation. We had anticipated that induction of a type 1 response to the 19-kDa antigen might confer some level of protection against *M. tuberculosis* challenge. However, it is clear from the results obtained in two different models that *M. vaccae* expressing the 19-kDa antigen not only confers no protective efficacy but that the infection is exacerbated in animals immunized with the recombinant in comparison to those receiving *M. vaccae* (vector control). Explanations to account for this unexpected finding fall into two general categories. It is possible that the existence of a type 1 response to the 19-kDa antigen has an adverse effect on the course of infection with *M. tuberculosis*. Alternatively, the presence of the 19-kDa antigen or an immune response to the 19-kDa antigen may alter the way in which the *M. vaccae* immunogen is processed, inhibiting induction of the protective effect of the control vaccine. Our results do not allow us to distinguish between these two possibilities. While it is clear that a type 1 response to the 19-kDa antigen is not in itself sufficient to confer protection, our results do not preclude a possible protective role for the 19-kDa antigen if *M. vaccae* was inoculated by a different route or if the recombinant was used in a different vector or as plasmid DNA. The ability to induce type 1 responses to *M. tuberculosis* proteins expressed from plasmid or cosmid constructs suggests that the recombinant *M. vaccae* system may provide a useful tool in the search for tuberculosis vaccine candidates.

ACKNOWLEDGMENTS

We thank Jelle Thole for helpful discussions and suggestions. We also thank John Belisle, Patrick Brennan, Uli Fruth, and Paul-Henri Lambert for their interest in the study. We are grateful to Åse Andersen and Arend Kolk for providing monoclonal antibodies HYT6 and F29-47.

This research was supported by grants from the National Institute of Health (AI35243) and the World Health Organization Programme for Vaccine Research and Development.

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Editor: S. H. E. Kaufmann