INFECTION AND IMMUNITY, Aug. 1998, p. 3643–3648 Vol. 66, No. 8
0019-9567/98/$04.00+0

An Epitope Delivery System for Use with Recombinant Mycobacteria

CHARLOTTE HETZEL,[1] RINY JANSSEN,[2] SARAH J. ELY,[2] NANNA M. KRISTENSEN,[2] KAREN BUNTING,[2,3] JONATHAN B. COOPER,[3] JONATHAN R. LAMB,[1]† DOUGLAS B. YOUNG,[2] AND JELLE E. R. THOLE[2]*

Department of Biology[1] and Department of Infectious Diseases and Microbiology,[2] Imperial College School of Medicine, London, and Biochemistry and Molecular Biology, School of Biological Sciences, University of Southampton, Southampton,[3] United Kingdom

Received 29 December 1997/Returned for modification 5 March 1998/Accepted 12 May 1998

We have developed a novel epitope delivery system based on the insertion of peptides within a permissive loop of a bacterial superoxide dismutase molecule. This system allowed high-level expression of heterologous peptides in two mycobacterial vaccine strains, *Mycobacterium bovis* bacille Calmette-Guérin (BCG) and *Mycobacterium vaccae*. The broader application of the system was analyzed by preparation of constructs containing peptide epitopes from a range of infectious agents and allergens. We report detailed characterization of the immunogenicity of one such construct, in which an epitope from the Der p1 house dust mite allergen was expressed in *M. vaccae*. The construct was able to stimulate T-cell hybridomas specific for Der p1, and it induced peptide-specific gamma interferon responses when used to immunize naive mice. This novel expression system demonstrates new possibilities for the use of mycobacteria as vaccine delivery vehicles.

Mycobacteria are potent immunogens, inducing both antibody and cell-mediated immune responses. BCG, an attenuated strain of *Mycobacterium bovis*, is the most widely used vaccine worldwide; *Mycobacterium tuberculosis* is routinely used for preparation of complete Freund's adjuvant; and components of the mycobacterial cell wall have been exploited in development of alternative adjuvant systems (4, 13, 23). With the advent of tools for genetic manipulation of mycobacteria, several reports have described the use of recombinant BCG as a potential vaccine vector (2, 19, 31, 32). A strain of BCG expressing the *ospA* antigen of *Borrelia burgdorferi* has been shown to induce protective immunity in animal models, for example (31).

With the aim of understanding factors underlying the immunogenicity of mycobacteria, and the eventual construction of improved mycobacterial vaccines, we wished to analyze the ability of mycobacteria to present a series of well-defined antigens to the immune system in experimental models. We have chosen initially to compare the immunogenicity of BCG, delivered as a live vaccine, with that of *Mycobacterium vaccae*, a soil organism that has been used as a killed preparation in human immunotherapy (9, 30). In initial experiments with a range of antigens, we were able to obtain only low-level expression and observed a tendency for decreased expression during subculture of recombinants. To obtain a high-level expression system suitable for use with different antigens, we have developed an epitope expression system based on the iron-containing superoxide dismutase (SOD) of *M. tuberculosis*.

M. tuberculosis SOD is a multimeric protein formed by the tight association of four 23-kDa subunits. SOD is located predominantly in the cytoplasm of mycobacteria but is also found in the extracellular fluid of cultures of slowly growing mycobacteria such as *M. tuberculosis* (3) and *Mycobacterium avium* (8). It was initially identified as a major target of the immune response in mice immunized with mycobacteria. The gene encoding the *M. tuberculosis* enzyme can be expressed at high levels in recombinant mycobacterial systems, accounting for as much as 10% of the total cell protein (11). High-level expression in *M. vaccae* has been exploited for protein purification, allowing establishment of the complete three-dimensional structure of the protein (6). Analysis of this structure identified an outward-extending loop which is distal from the reactive site of the enzyme and is not involved in subunit interactions. In the present study, we describe a strategy for epitope expression in mycobacteria which is based on targeting of this loop as an insertion site for foreign peptides.

MATERIALS AND METHODS

Epitope carrier vector construction. A *Bam*HI site was created in the *sodA* gene, present on plasmid p16R1/SOD (11), using two-step PCR-directed mutagenesis. The resulting plasmid, designated p23.1, contains a unique *Bam*HI site between codons 51 and 53 of the *sodA* gene. Complementary oligonucleotides flanked by *Bam*HI sites were subsequently used to introduce immunodominant peptides from various antigens (Table 1). In each case, codon usage was optimized for that of known *M. tuberculosis* proteins. Constructs were routinely checked by DNA sequence analysis.

Bacterial cultures and transformation. *Escherichia coli* DH5α was grown at 37°C in LB medium. *M. vaccae* NCTC 11659 (supplied by John Stanford, University College and Middlesex Hospital School of Medicine, London, United Kingdom) was grown in Middlebrook 7H9 medium supplemented with 2% glucose. *M. bovis* BCG (strain P3) was grown in Middlebrook 7H9 medium supplemented with albumin, dextrose, and catalase as recommended by the manufacturer (Difco, West Molesey, United Kingdom). When appropriate, hygromycin B (Sigma, Northampton, United Kingdom) was added at 200 μg/ml for *E. coli* or at 50 μg/ml for *M. vaccae* and BCG (13). Transformation of mycobacteria was carried out by electroporation (11), whereas *E. coli* was transformed by using standard procedures (27). For immunization purposes, *M. vaccae* recombinants expressing chimeric SOD proteins were grown on 7H11 plates supplemented with 2% glucose and 50 μg of hygromycin per ml.

Analysis of expression of chimeric SOD proteins. For analysis of protein expression, sonicated extracts of recombinant *M. vaccae* or BCG were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) (22) and Western blotting. Blots were developed with a monoclonal antibody (D2D) against mycobacterial SOD (34) and visualized by chemiluminescence (Amersham International, Amersham, United Kingdom). The en-

* Corresponding author. Present address: TNO Prevention and Health, Zernikedreef 9, P.O. Box 2215, 2301 CE Leiden, The Netherlands. Phone: 3171-5181276. Fax: 3171-5181901. E-mail: j.thole@pg.tno.nl.

† Present address: Respiratory Medicine Unit, University of Edinburgh Medical School, Edinburgh, United Kingdom.

TABLE 1. Constructs used

Construct	Peptide sequence	Origin	Reference(s)	Length (residues)	Expression
SOD					+
SOD-p1	111–139 of Der p1	House dust mite	16, 20	29	+
SOD-p2	87–105 of Der p2	House dust mite	17	14	++
SOD-OVA	323–339 of ovalbumin		29	17	++
SOD-HA	126–138 of hemagglutinin	Influenza virus	28	22	+++
SOD-NP	366–374 of nucleoprotein	Influenza virus	33	9	+++
SOD-M2	58–66 of matrix protein	Influenza virus	1	9	+++
SOD-48	307–319 of hemagglutinin	Influenza virus	26	13	++
SOD-3.20	335–349 of nucleoprotein	Influenza virus	33	15	++
SOD-45.1	380–391 of nucleoprotein	Influenza virus	18	12	++
SOD-65	1–20 of Hsp65	*M. tuberculosis*	12	20	+
SOD-40.6	Various epitopes[a]	Plasmodium	15	30	+
SOD-36.8	Various epitopes[b]	Plasmodium	15	60	±
SOD-RSV	82–90 of 22-kDa protein	Respiratory syncytial virus	21	9	+++

[a] CD8 epitopes of STARP and LSA-1 and a CD4 epitope of CS protein (15).
[b] CD8 epitopes of STARP, TRAP, CS protein, and LSA-1 and a CD4 epitope of CS protein (15).

zymatic activity of recombinant SOD proteins was examined by activity staining of gels run under nondenaturing conditions (36).

Preparation of antigens. Aliquots of 100 mg/ml of recombinant mycobacterial suspensions were frozen at −70°C in 15% glycerol. For immunization, the glycerol was washed away, the bacteria were heat-killed (20 min, 80°C) where necessary, and the preparations were resuspended at the appropriate concentration in phosphate-buffered saline–0.05% Tween 80. Soluble native *M. vaccae* antigens for in vitro stimulation were prepared by sonicating the bacterial suspensions in phosphate-buffered saline and filtering them through a 0.22-μm-pore-size filter. Peptide 111–139 of Der p1 (sequence, FGISNYCQIYPPNANK IREALAQTHSAIA [5]) and peptide 111–119 were made on an ABIMED 422 synthesizer (ABIMED, Langenfeld, Germany) by the simultaneous peptide synthesis method. The purity of the peptide was verified by reverse-phase C_{18} high-pressure liquid chromatography (Lichrospher; Merck, Darmstadt, Germany) and was shown to be routinely over 75%.

Animals and immunization protocols. Female C57BL/6J (*H-2*^{b}) mice (6 to 8 weeks old) from Harlan-Olac (Bicester, United Kingdom) were used in the study. Mice, usually in groups of four, were immunized subcutaneously (s.c.) in the flank with preparations of 1 to 1,000 μg of *M. vaccae* (corresponding to 10^6 to 10^9 organisms) in a 200-μl volume.

Cell culture. Single-cell suspensions of splenocytes or lymph node cells were cultured in 96-flat-well microtiter plates (Nunc, Roskilde, Denmark) at 2×10^6 cells/ml in RPMI 1640 supplemented with 5% fetal calf serum, 2 mM L-glutamine, 20 IU of penicillin and 20 μg of streptomycin (Gibco, Grand Island, N.Y.) per ml, and 0.05 μM 2-mercaptoethanol (Sigma, St. Louis, Mo.) (200 μl/well) at 37°C in humidified air containing 5% CO_2. Cells were incubated in triplicate wells alone or with various concentrations of antigens for the assessment of gamma interferon (IFN-γ) in supernatants at 72 h. Proliferation was measured by pulsing for the last 6 h with [^{3}H]thymidine (0.5 μCi/well; Amersham), harvesting the contents of each well onto glass fiber mats, and determining the incorporation of ^{3}H in a Betaplate liquid scintillation counter (Wallac, Turku, Finland).

The isolation of Der p1-specific T-cell hybridomas has been previously described (20). Briefly, the hybridomas were generated by polyethylene glycol (Sigma)-induced fusion of Der p1-specific CD4$^+$ T-cell lines derived from *H-2*^{b} mice with the BW 5147 fusion partner (a kind gift from H. Bodmer, Nuffield Department of Clinical Medicine, Oxford, United Kingdom) and selection with hypoxanthine-aminopterin-thymidine medium (Sigma). Hybridomas recognizing epitopes between residues 111 and 139 of Der p1 were used in this study. Activation of the hybridomas was measured by culturing them at 5×10^4 cells/well with appropriate antigens and 10^5 irradiated (2,500 rads) syngeneic splenocytes as antigen-presenting cells (APC) for 20 h and assaying the interleukin-2 (IL-2) content of the culture supernatants.

Cytokine assays. IFN-γ was measured by enzyme-linked immunosorbent (ELISA) using the rat anti-mouse coating antibody R4-6A2 and biotinylated detector antibody XMG1.2 pair (PharMingen, San Diego, Calif.). The binding of biotinylated antibody was detected with alkaline phosphatase-conjugated streptavidin (Amersham) followed by *p*-nitrophenylphosphate (Sigma) at 1 mg/ml in Tris HCl buffer (pH 9.6) as the substrate. Optical density at 405 nm of the product was measured with a Bio-rad ELISA reader. Recombinant murine IFN-γ (PharMingen) was used to construct a standard curve.

IL-2 was measured in supernatants from the IL-2-dependent cell line CTLL-2. Briefly, 5×10^3 CTLL-2 (American Type Culture Collection) cells in 50 μl were incubated with 50 μl of test supernatant in triplicate for 24 h, and proliferation was measured over the last 6 h by pulsing with [^{3}H]thymidine (0.5 μCi/well; Amersham), harvesting the contents of each well onto glass fiber mats, and determining the incorporation of ^{3}H in a Betaplate liquid scintillation counter (Wallac).

ELISA for specific antibodies. Der p1 peptide 111–139 (5 μg/ml) was coated onto Maxisorp microtiter plates (Nunc) in bicarbonate coating buffer (Sigma) overnight at 4°C. After blocking (1 h, Tris-buffered saline–1% bovine serum albumin) and washing, serum dilutions were incubated in duplicate for 2 h at 37°C. After washing, the amount of specific immunoglobulin (IgG) isotypes bound was detected with alkaline phosphatase-conjugated rat anti-mouse IgG1 and IgG2a (PharMingen) for 1 h at 37°C. The enzyme substrate *p*-nitrophenylphosphate was added, and the soluble product was measured as described for the cytokine ELISAs.

Isolation of CD4$^+$ and CD8$^+$ cells. CD4$^+$ and CD8$^+$ T cells were isolated from lymph node cells from immunized mice by immunomagnetic selection. After incubation with biotinylated anti-CD4 (GK1.5; PharMingen) or anti-CD8 (clone SK1; Becton Dickinson, Oxford, United Kingdom), cells were incubated with streptavidin-conjugated microbeads (Miltenyi Biotec) (25) and selected by using the MACS system (Miltenyi Biotec). Positively isolated fractions were collected, and the efficiency of the separation was cross-checked by staining with rat anti-mouse CD4 conjugated to phycoerythrin (CT-CD4; Caltag, Burlingame, Calif.), rat anti-mouse CD8 conjugated to phycoerythrin (clone 53-6.7; Sigma), or fluorescein isothiocyanate-conjugated streptavidin (Amersham). Samples containing 10^5 cells from each of the stained fractions were analyzed on a Becton Dickinson FACScan flow cytometer.

RESULTS

Construction and expression of chimeric SOD proteins. To exploit the adjuvant activities of mycobacteria, a mycobacterial antigen delivery system for high-level expression of epitopes was developed by using the tetrameric SOD protein of *M. tuberculosis* as a carrier. To incorporate short peptide sequences into SOD, we targeted an outward-extending loop (positions 52 to 59 [Fig. 1]) between two alpha helices in the N-terminal domain (7) for several reasons. First, this domain does not seem to be involved in the enzymatic activity or in the interaction between the SOD monomers; second, the region around amino acid 53 appears to be particularly variable between different mycobacterial species, suggesting that variation in amino acid sequence in this region does not influence the structural and functional properties of the SOD molecule. We constructed a plasmid (p23.1) in which a unique *Bam*HI site was introduced between codons 51 and 53 of *M. tuberculosis* SOD. Expression of SOD was under the control of its native *M. tuberculosis* promoter, and the plasmid contained a mycobacterial origin of replication. This vector was subsequently used for the insertion of a variety of peptides derived from heterologous antigens listed in Table 1.

Constructs were introduced into *M. vaccae* or BCG, and expression was monitored by Coomassie brilliant blue staining,

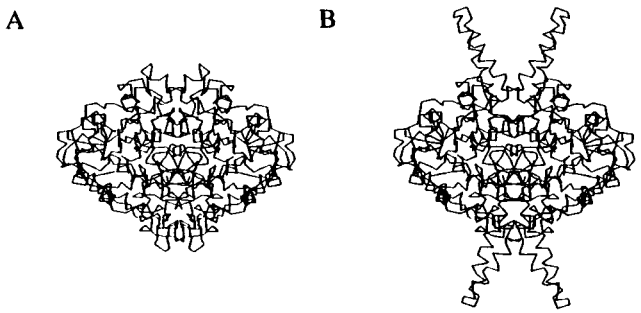

FIG. 1. Identification of a permissive loop in *M. tuberculosis* SOD. Inspection of the crystal structure of the tetrameric *M. tuberculosis* SOD (A) identifies an outward-extending loop centered on amino acid residue 53. For epitope expression, this region of the gene was engineered to contain a *Bam*HI site. Oligonucleotides encoding appropriate epitopes were inserted at this site to construct a series of chimeric SOD molecules. The structure of the one of the chimeric SOD proteins, SOD-HA, containing peptide 126–138 of influenza virus HA, was predicted via computer modelling (B) using the known structure of SOD and the known structure of HA. The modelling was done with QUANTA (Molecular Simulations Incorporated), using conventional loop-database search to find homologous fragments with the appropriate distance-geometry to fit the framework of the protein. The core SOD protein (Fig. 1A) is also present in the predicted chimeric SOD-HA protein (Fig. 1B), and the inserted epitope is in an extended loop far from functional sites of the protein.

Western blotting, and enzyme activity. Figure 2A illustrates expression of two chimeric SOD proteins in *M. vaccae* extracts stained for total protein. The chimeric SOD proteins are among the most prominent bands in the extracts, although the expression levels are lower than that of the wild-type SOD protein (lane 1). Densitometric scanning of gels after SDS-PAGE indicated that the bands representing the chimeric SOD proteins correspond to 2 to 10% of the total protein in *M. vaccae* extracts.

The level of expression of recombinant proteins in BCG was lower than that in *M. vaccae* and was visualized by Western blot analysis using a monoclonal antibody specific for SOD. Figures 2B and C compare the level of expression of control SOD and three chimeric constructs in BCG and in *M. vaccae*. In each case, the amount of recombinant protein was approximately fivefold higher in *M. vaccae*. The 23-kDa protein seen in all lanes of Fig. 2B and C corresponds to the native SOD enzyme present in BCG and in *M. vaccae*, respectively. Introduction of the same constructs into another rapidly growing mycobacterium, the laboratory strain of *M. smegmatis*, resulted in levels of expression similar to those observed in BCG (data not shown).

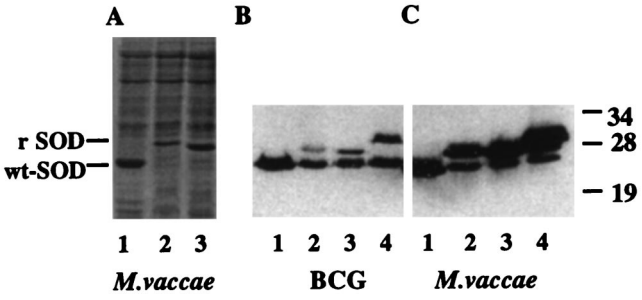

FIG. 2. Expression of chimeric SOD molecules. (A) Total protein analysis. Sonicates from *M. vaccae* recombinants expressing wild-type SOD (wt-SOD; lane 1), SOD-p1 (lane 2), and SOD-p2 (lane 3) were fractionated by SDS-PAGE, and the gel was stained with Coomassie brilliant blue. Recombinant (r) SOD proteins are seen as major bands. (B and C) Western blot analysis. Extracts from BCG (B) and *M. vaccae* recombinants (C) were separated by SDS-PAGE, transferred to nitrocellulose, and stained with a monoclonal antibody to SOD. Lane 1, wild-type SOD; lane 2, SOD-p1; lane 3, SOD-p2; lane 4, SOD-HA. The endogenous SOD from BCG and *M. vaccae* SOD is seen in all lanes (lower band). Sizes are indicated in kilodaltons.

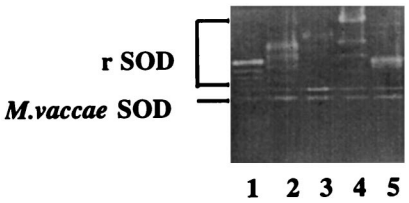

FIG. 3. SOD activity in nondenaturing gels. Sonicates from *M. vaccae* recombinants were run on nondenaturing gels and stained for SOD enzyme activity. Native SOD activity from *M. vaccae* is seen as the two lower bands present in all lanes. The variable upper bands represent a series of hybrid tetramers formed by the recombinant (r) SOD. Lane 1, wild-type SOD; lane 2, SOD-OVA; lane 3, SOD-p1; lane 4, SOD-p2; lane 5, SOD-HA.

Assay of SOD activity in nondenaturing gels revealed that the chimeric proteins were expressed as functional enzymes (Fig. 3). As reported previously (35), expression of the wild-type *M. tuberculosis* SOD in *M. vaccae* generated the *M. tuberculosis* tetramer (the predominant top band seen in lane 1) as well as a series of hybrid enzymes containing different combinations of *M. tuberculosis* and *M. vaccae* subunits. A similar pattern was seen with the chimeric proteins, although differences in size and charge led to altered mobility of the tetramers during gel electrophoresis. These data suggest that the incorporation of epitopes in the extended loop of SOD is tolerated without having a major effect on enzyme activity or on the ability to form tetramers. The structure of one of the SOD chimera was predicted by computer modelling. Since the crystal structure of hemagglutinin (HA) of influenza virus is known, the SOD-HA protein was chosen. Consistent with the experimental data, comparison of the known structure of SOD (Fig. 1A) with the predicted structure of SOD-HA (Fig. 1B) revealed that the folding of the active SOD core of the SOD-HA protein is identical to that of the wild-type protein.

Antigenicity of the recombinant *M. vaccae*. Since mycobacteria are potent inducers of cell-mediated immunity, we wished to study T-cell responses to epitopes delivered by mycobacteria as chimeric SOD proteins. Therefore, we focused on a construct that contains an immunodominant region from Der p1 (residues 111 to 139), one of the major allergens of house dust mites, which encompasses well-characterized murine major histocompatibility complex (MHC) class I- and class II-restricted T-cell epitopes (residues 111 to 119 and 113 to 127, respectively) (14, 16, 17, 20). The antigenicity of the chimeric SOD protein containing Der p1 (111–139) expressed in *M. vaccae* (Mv-p1) was examined by measuring its ability to stimulate two different murine $CD4^+$ T-cell hybridomas recognizing epitopes within the region from residues 111 to 139 of Der p1. Both hybridoma 1BB8 (Fig. 4) and hybridoma AD2 (results not shown) were activated by the presence of intact Mv-p1 and, to an even greater extent, by sonicated Mv-p1. In contrast, preparations of control *M. vaccae* expressing wild-type SOD (Mv-SOD) failed to stimulate any IL-2 production. These findings confirmed that the peptide expressed by Mv-p1 was in a form that could be recognized by peptide 111–139-specific murine T cells. Comparison of the response to sonicated Mv-p1 and to peptide and estimation of the amount of peptide in such a sonicate revealed that the antigenicity of a peptide delivered by *M. vaccae* is similar to that of the peptide itself.

Immunogenicity of recombinant *M. vaccae*. The immunogenicity of Mv-p1 was tested by s.c. immunization of C57BL/6J

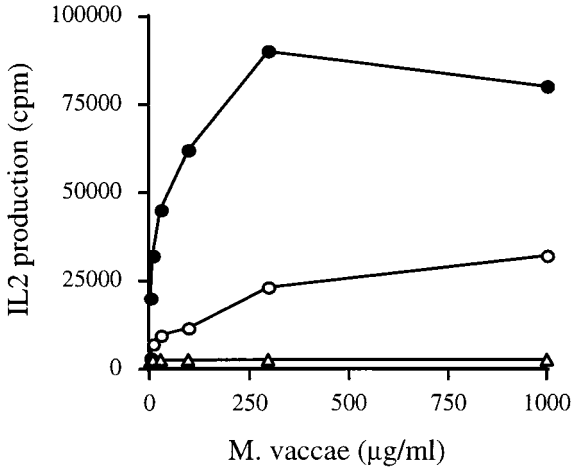

FIG. 4. Recognition of *M. vaccae* recombinants by T-cell hybridoma. Murine T-cell hybridoma 1BB8 specific for Der p1(111–139) was cultured in the presence of APC with sonicated or intact heat-killed Mv-p1 (filled and open circles, respectively) and sonicated Mv-SOD (open triangles), and supernatants were tested for IL-2, using the CTTL-2 cell line. Only the recombinant expressing the specific peptide stimulated the cells. For comparison, supernatant from the same hybridoma stimulated with 0.1 μM synthetic peptide produced 93×10^3 cpm in the IL-2 assay.

mice with 5×10^8 (500 μg) heat-killed Mv-p1 bacilli without adjuvant. A heat-killed preparation was used for two reasons. First, heat-killed *M. vaccae* is clinically applicable in humans, and second, *M. vaccae* is not believed to survive after immunization, suggesting no extra benefit for the use of viable bacteria. Five days later, draining lymph nodes were isolated and T-cell responses were evaluated by measuring IFN-γ production and proliferation in response to in vitro stimulation with peptide 111–139. Immunization with Mv-p1 resulted in the production of high levels of peptide-specific IFN-γ by lymph node cells in culture (Fig. 5A) and peptide-specific proliferation (data not shown). This finding demonstrates that the peptide is immunogenic when presented in vivo as an SOD chimera delivered by *M. vaccae*. A control immunization with 5×10^8 heat-killed Mv-SOD did not result in peptide-specific IFN-γ responses. However, when a soluble extract of *M. vaccae* was used as an antigen, an equally strong IFN-γ response was observed in mice immunized with either Mv-p1 or Mv-SOD.

Peptide 111–139 of Der p1 can also be recognized by B cells. Therefore, mice were immunized twice with a 3-week interval with 5×10^8 heat-killed or Mv-p1 or with control Mv-SOD. Serum samples were collected 10 days after boost and tested for the presence of peptide 111–139-specific IgG1 and IgG2a. No IgG1 was detectable. IgG2a titers between 30 and 100 were measured in mice that received Mv-p1. No peptide-specific antibodies were detectable in sera derived from control immunized mice. This finding indicates that it is possible to induce antibody responses by using the mycobacterial system for the delivery of this particular peptide, although this induction is very inefficient.

$CD4^+$ versus $CD8^+$ T cells as the source of IFN-γ. Since peptide 111–139 of Der p1 contains both a CD4 and a CD8 epitope, the cell type responsible for peptide-induced IFN-γ production was studied in more detail. CD4- and CD8-enriched cell populations were prepared by immunomagnetic fractionation of lymph node cells from mice inoculated 5 days previously with 5×10^8 heat-killed Mv-p1. By flow cytometry, the proportion of $CD4^+$ cells in the $CD8^+$ fraction and the proportion of $CD8^+$ cells in the $CD4^+$ fraction were routinely less than 2%. As shown in Fig. 5B, culture with Der p1 peptide resulted in production of IFN-γ by unfractionated lymph node cells and by $CD4^+$ cells (in the presence of syngeneic APC). No IFN-γ was produced by $CD8^+$ cells incubated with Der p1 peptide 111–139 or with peptide 111–119, which encompasses the CD8 epitope.

DISCUSSION

The expression of foreign antigens in recombinant mycobacteria is attractive as a strategy for vaccine production and more generally as an approach to probing mycobacterial interactions with the immune system. The fact that exogenous proteins are often expressed at low levels in mycobacteria imposes an important limitation on development of this approach. We have explored a peptide expression system in order to increase the

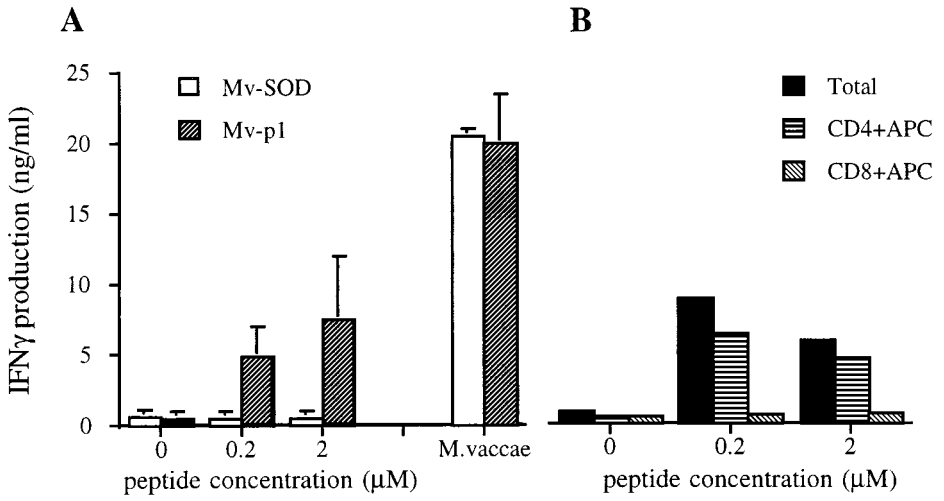

FIG. 5. Immunogenicity of *M. vaccae* recombinants. Mice were immunized s.c. once with Mv-p1 or Mv-SOD, and antigen-specific cytokine responses were monitored 5 days later in inguinal lymph node cells. (A) Lymph node cells (4×10^5 cells/well) from mice immunized with Mv-p1 produced IFN-γ when incubated with specific peptide or with *M. vaccae* extract (2.5 μg protein/well). Cells from mice immunized with control Mv-SOD responded only to the *M. vaccae* antigen. (B) Lymph node cells were fractionated into $CD4^+$ and $CD8^+$ populations and incubated (2×10^5 cells/well plus 3×10^5 irradiated APC/well) in the presence or absence of specific peptide. IFN-γ production was observed only with the $CD4^+$ population.

repertoire of epitopes that can be delivered by mycobacteria. We selected a native mycobacterial protein, SOD from *M. tuberculosis*, as a carrier protein based on its high level of expression and known crystal structure. While differences were observed in the level of expression of different peptides (Table 1), the SOD expression system was found to be broadly applicable to a wide range of epitopes, and we have been able to express chimeric SOD proteins containing as many as 60 amino acids. The success of this expression system is probably due to the inherent high level of expression and stability of the SOD molecule and the fact that the point of peptide insertion does not significantly disrupt assembly of the functionally active tetrameric enzyme. Expression of all tested chimeric proteins was higher in *M. vaccae* than in BCG, an effect that could be due to differences in promoter activity, plasmid copy number, or proteolytic activity between the two strains. In addition, expression of the endogenous *M. vaccae* and BCG SOD proteins in the two strains might interfere with expression of the plasmid-encoded chimeric SOD to differing extents.

A construct in which an immunodominant peptide from a major house dust mite allergen (Der p1) was expressed as a chimeric SOD protein in *M. vaccae* was selected for detailed immunological characterization. Intact or sonicated bacteria were recognized by murine T-cell hybridomas specific for the peptide, demonstrating that the chimeric SOD is available for antigen processing and presentation. More efficient recognition of sonicated extracts suggests that there may be differences in processing between the intact and disintegrated mycobacteria. These differences could be related to an inability of irradiated spleen cells to process intact mycobacteria, or perhaps to a difference in kinetics of processing. Immunization experiments demonstrated that recombinant mycobacteria were also able to induce a specific T-cell response to the Der p1 peptide in vivo, as assessed by IFN-γ production by lymph node cells. Since peptide 111–139 contains both a CD4 and a CD8 epitope (14), T-cell separation experiments were performed to determine the phenotype of the responding cells. These experiments, together with cytotoxicity assays (not shown), demonstrated the response to be exclusively attributable to $CD4^+$ T cells, suggesting that antigen presentation occurs only via MHC class II molecules.

Experiments with transgenic mice lacking the β2-microglobulin gene have demonstrated that T-cell responses restricted by MHC class I molecules play an important role in protection against *M. tuberculosis* infection (10), and recent in vitro experiments suggest that mycobacterial species differ in the ability to deliver antigens by this route (24). An important application of the expression system described here is that it will allow us to compare the ability of different mycobacteria to deliver the same antigen to the immune system. We are currently evaluating the immunogenicity of the recombinant BCG constructs with a view to comparison with *M. vaccae* and in some cases with virulent *M. tuberculosis*. This strategy provides an approach to identifying microbial factors involved in immunogenicity and may also allow us to select vaccine candidates with particular immunological profiles. We are also using a peptide approach to analyze the immunogenicity of an identical epitope expressed in a cytoplasmic (SOD) compared to a membrane-associated carrier protein in mycobacteria. Another application of this mycobacterial expression system is the use of such recombinants for immunomodulation. As mycobacteria are especially capable of inducing Th1 cell development, characterized by the production of high levels of IFN-γ and IL-2, it may be possible to use constructs expressing epitopes of allergens for modulation of allergen-specific Th2 cell responses, characterized by the production of high levels of IL-4 and IL-5. However, for this purpose the exact nature of T-cell responses raised with recombinant mycobacteria expressing epitopes of allergens has to be evaluated in more detail.

Diversity in the repertoire of peptides presented by different MHC molecules represents an obvious limitation to an epitope-based approach to therapeutic vaccine development. In the SOD expression system, it may be possible to address this problem in part by insertion of multiple epitopes within a single chimeric construct. The repertoire could be expanded further by expressing tetrameric proteins comprised of different recombinant monomers. For broader application, the epitope system could be used for initial experimental identification of a mycobacterial host strain with appropriate immunogenic properties, with a view to subsequent optimization of specific strategies for stable expression of each whole antigen molecule.

ACKNOWLEDGMENTS

We thank the EC, the Royal Society, the MRC, the Wellcome Trust, and the British Lung Foundation for financial support.

C. Hetzel and R. Janssen made equivalent contributions to this study.

REFERENCES

1. **Adler, S., R. Frank, A. Lanzavecchia, and S. Weiss.** 1994. T cell epitope analysis with peptides simultaneously synthesized on cellulose membranes: fine mapping of two DQ dependent epitopes. FEBS Lett. **352:**167–170.
2. **Aldovini, A., and R. A. Young.** 1991. Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. Nature **351:**479–482.
3. **Andersen, P., D. Askgaard, A. Gottschau, J. Bennedsen, S. Nagai, and I. Heron.** 1992. Identification of immunodominant antigens during infection with *Mycobacterium tuberculosis*. Scand. J. Immunol. **36:**823–831.
4. **Barnes, P. F., D. Chatterjee, J. S. Abrams, S. Lu, E. Wang, M. Yamamura, P. J. Brennan, and R. L. Modlin.** 1992. Cytokine production induced by *Mycobacterium tuberculosis* lipoarabinomannan. Relationship to chemical structure. J. Immunol. **149:**541–547.
5. **Chua, K. Y., P. K. Kehal, and W. R. Thomas.** 1993. Sequence polymorphisms of cDNA clones encoding the mite allergen Der p I. Int. Arch. Allergy Immunol. **101:**364–368.
6. **Cooper, J. B., H. P. Driessen, S. P. Wood, Y. Zhang, and D. Young.** 1994. Crystallization and preliminary X-ray analysis of the superoxide dismutase from *Mycobacterium tuberculosis*. J. Mol. Biol. **235:**1156–1158.
7. **Cooper, J. B., K. McIntyre, M. O. Badasso, S. P. Wood, Y. Zhang, T. R. Garbe, and D. Young.** 1995. X-ray structure analysis of the iron-dependent superoxide dismutase from *Mycobacterium tuberculosis* at 2.0 Angstroms resolution reveals novel dimer-dimer interactions. J. Mol. Biol. **246:**531–544.
8. **Escuyer, V., N. Haddad, C. Frehel, and P. Berche.** 1996. Molecular characterization of a surface-exposed superoxide dismutase of *Mycobacterium avium*. Microb. Pathog. **20:**41–55.
9. **Etemadi, A., R. Farid, and J. L. Stanford.** 1992. Immunotherapy for drug-resistant tuberculosis. Lancet **340:**1360–1361.
10. **Flynn, J. L., M. M. Goldstein, K. J. Triebold, B. Koller, and B. R. Bloom.** 1992. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. Proc. Natl. Acad. Sci. USA **89:**12013–12017.
11. **Garbe, T. R., J. Barathi, S. Barnini, Y. Zhang, C. Abou-Zeid, D. Tang, R. Mukherjee, and D. B. Young.** 1994. Transformation of mycobacterial species using hygromycin resistance as selectable marker. Microbiology **140:**133–138.
12. **Geluk, A., W. Bloemhoff, R. R. de Vries, and T. H. Ottenhoff.** 1992. Binding of a major T cell epitope of mycobacteria to a specific pocket within HLA-DRw17(DR3) molecules. Eur. J. Immunol. **22:**107–113.
13. **Gupta, R. K., and G. R. Siber.** 1995. Adjuvants for human vaccines—current status, problems and future prospects. Vaccine **13:**1263–1276.
14. **Harris, S. J., J. F. Roth, N. Savage, S. A. Woodrow, I. K. Hemingway, G. F. Hoyne, J. R. Lamb, and G. T. Layton.** 1997. Prediction of murine MHC class I epitopes in a major house dust mite allergen and induction of T1-type CD8+ T cell responses. Int. Immunol. **9:**273–280.
15. **Hill, A. V., J. Elvin, A. C. Willis, M. Aidoo, C. E. Allsopp, F. M. Gotch, X. M. Gao, M. Takiguchi, B. M. Greenwood, and A. R. Townsend.** 1992. Molecular analysis of the association of HLA-B53 and resistance to severe malaria. Nature **360:**434–439.
16. **Hoyne, G. F., M. G. Callow, M. C. Kuo, and W. R. Thomas.** 1993. Comparison of antigen presentation by lymph node cells from protein and peptide-primed mice. Immunology **78:**58–64.

17. **Hoyne, G. F., M. G. Callow, M. C. Kuo, and W. R. Thomas.** 1993. Characterization of T-cell responses to the house dust mite allergen Der p II in mice. Evidence for major and cryptic epitopes. Immunology **78:**65–73.
18. **Huet, S., D. F. Nixon, J. B. Rothbard, A. Townsend, S. A. Ellis, and A. J. McMichael.** 1990. Structural homologies between two HLA B27-restricted peptides suggest residues important for interaction with HLA B27. Int. Immunol. **2:**311–316.
19. **Jacobs, W. R., Jr., S. B. Snapper, L. Lugosi, and B. R. Bloom.** 1990. Development of BCG as a recombinant vaccine vehicle. Curr. Top. Microbiol. Immunol. **155:**153–160.
20. **Kristensen, N. M., G. F. Hoyne, J. D. Hayball, C. Hetzel, T. Bourne, and J. R. Lamb.** 1996. Induction of T cell responses to the invariant chain derived peptide CLIP in mice immunized with the group 1 allergen of house dust mite. Int. Immunol. **8:**1091–1098.
21. **Kulkarni, A. B., H. C. Morse III, J. R. Bennink, J. W. Yewdell, and B. R. Murphy.** 1993. Immunization of mice with vaccinia virus-M2 recombinant induces epitope-specific and cross-reactive Kd-restricted $CD8^+$ cytotoxic T cells. J. Virol. **67:**4086–4092.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature **227:**680–685.
23. **Lidgate, D. M., and N. E. Byars.** 1995. Development of an emulsion-based muramyl dipeptide adjuvant formulation for vaccines. Pharm. Biotechnol. **6:**313–324.
24. **Mazzaccaro, R. J., M. Gedde, E. R. Jensen, H. M. van Santen, H. L. Ploegh, K. L. Rock, and B. R. Bloom.** 1996. Major histocompatibility class I presentation of soluble antigen facilitated by *Mycobacterium tuberculosis* infection. Proc. Natl. Acad. Sci. USA **93:**11786–11791.
25. **Miltenyi, S., W. Muller, W. Weichel, and A. Radbruch.** 1990. High gradient magnetic cell separation with MACS. Cytometry **11:**231–238.
26. **Rothbard, J. B., R. I. Lechler, K. Howland, V. Bal, D. D. Eckels, R. Sekaly, E. O. Long, W. R. Taylor, and J. R. Lamb.** 1988. Structural model of HLA-DR1 restricted T cell antigen recognition. Cell **52:**515–523.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Scott, B., R. Liblau, S. Degermann, L. A. Marconi, L. Ogata, A. J. Caton, H. O. McDevitt, and D. Lo.** 1994. A role for non-MHC genetic polymorphism in susceptibility to spontaneous autoimmunity. Immunity **1:**73–83.
29. **Shimonkevitz, R., S. Colon, J. W. Kappler, P. Marrack, and H. M. Grey.** 1984. Antigen recognition by H-2-restricted T cells. II. A tryptic ovalbumin peptide that substitutes for processed antigen. J. Immunol. **133:**2067–2074.
30. **Stanford, J. L., and J. M. Grange.** 1994. The promise of immunotherapy for tuberculosis. Respir. Med. **88:**3–7.
31. **Stover, C. K., G. P. Bansal, M. S. Hanson, J. E. Burlein, S. R. Palaszynski, J. F. Young, S. Koenig, D. B. Young, A. Sadziene, and A. G. Barbour.** 1993. Protective immunity elicited by recombinant bacille Calmette-Guerin (BCG) expressing outer surface protein A (OspA) lipoprotein: a candidate Lyme disease vaccine. J. Exp. Med. **178:**197–209.
32. **Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, and G. F. Hatfull.** 1991. New use of BCG for recombinant vaccines. Nature **351:**456–460.
33. **Townsend, A. R., J. Rothbard, F. M. Gotch, G. Bahadur, D. Wraith, and A. J. McMichael.** 1986. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell **44:**959–968.
34. **Young, D. B., M. J. Fohn, S. R. Khanolkar, and T. M. Buchanan.** 1985. Monoclonal antibodies to a 28,000 mol. wt protein antigen of *Mycobacterium leprae*. Clin. Exp. Immunol. **60:**546–552.
35. **Zhang, Y., B. Heym, B. Allen, D. Young, and S. Cole.** 1992. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. Nature **358:**591–593.
36. **Zhang, Y., R. Lathigra, T. Garbe, D. Catty, and D. Young.** 1991. Genetic analysis of superoxide dismutase, the 23 kilodalton antigen of *Mycobacterium tuberculosis*. Mol. Microbiol. **5:**381–391.

Editor: R. N. Moore