Induction of Neutralizing Antibodies against Diphtheria Toxin by Priming with Recombinant *Mycobacterium bovis* BCG Expressing CRM<sub>197</sub>, a Mutant Diphtheria Toxin

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BCG, the attenuated strain of *Mycobacterium bovis*, has been widely used as a vaccine against tuberculosis and is thus an important candidate as a live carrier for multiple antigens. With the aim of developing a recombinant BCG (rBCG) vaccine against diphtheria, pertussis, and tetanus (DPT), we analyzed the potential of CRM<sub>197</sub>, a mutated nontoxic derivative of diphtheria toxin, as the recombinant antigen for a BCG-based vaccine against diphtheria. Expression of CRM<sub>197</sub> in rBCG was achieved using *Escherichia coli*-mycobacterium shuttle vectors under the control of pBlAφ*, an upregulated β-lactamase promoter from *Mycobacterium fortuitum*. Immunization of mice with rBCG-CRM<sub>197</sub> elicited an anti-diphtheria toxoid antibody response, but the sera of immunized mice were not able to neutralize diphtheria toxin (DTx) activity. On the other hand, a sub-immunizing dose of the conventional diphtheria-tetanus vaccine, administered in order to mimic an infection, showed that rBCG-CRM<sub>197</sub> was able to prime the induction of a humoral response within shorter periods. Interestingly, the antibodies produced showed neutralizing activity only when the vaccines had been given as a mixture in combination with rBCG expressing tetanus toxin fragment C (FC), suggesting an adjuvant effect of rBCG-FC on the immune response induced by rBCG-CRM<sub>197</sub>. Isotype analysis of the anti-diphtheria toxoid antibodies induced by the combined vaccines, but not rBCG-CRM<sub>197</sub> alone, showed an immunoglobulin G1-dominant profile, as did the conventional vaccine. Our results show that rBCG expressing CRM<sub>197</sub> can elicit a neutralizing humoral response and encourage further studies on the development of a DPT vaccine with rBCG.

Many currently used vaccines require multiple doses to achieve maximum protection, which has led to reduced coverage of vaccination campaigns, especially in developing countries. The use of live viral or bacterial carriers for heterologous antigen presentation, such as vaccinia virus, *Salmonella*, and *Mycobacterium bovis* BCG (Bacille Calmette-Guérin), has been intensively investigated in an effort to reduce the number of doses required for immunization. *M. bovis* BCG is the most widely used live vaccine, having been administered as an antituberculosis vaccine to over 3 billion individuals. It has several features that have encouraged its use as a live carrier for recombinant antigens, such as low production cost, possibility of administration at birth with very strong adjuvant activity, induction of immunity after a single dose, and low frequency of side effects. The induction of humoral and cellular immune responses against antigens from several pathogens by recombinant BCG (rBCG) strains has been reported, such as rBCG expressing antigens from human immunodeficiency virus (25, 29), simian immunodeficiency virus (30), *Leishmania major* (1, 6), *Plasmodium falciparum* (13), *Streptococcus pneumoniae* (18), and *Borrelia burgdorferi* (26).

The conventional diphtheria-pertussis-tetanus (DPT) vaccine was shown to be extremely efficient, and the recently developed acellular DPT vaccine showed lower reactogenicity. However, both DPT and acellular DPT vaccines require multiple doses to attain complete protection, and the acellular DPT vaccine is expensive. The expression of DPT antigens in live carriers such as BCG could thus provide a single-dose vaccine against these pathogens. Tetanus and pertussis antigens have been expressed in rBCG, inducing significant immune responses (2, 5, 21), but expression of diphtheria antigens in an rBCG vaccine has not yet been described.

Diphtheria toxin (DTx) is a secreted molecule of 58.35 kDa produced by *Corynebacterium diphtheriae* and composed of two functional subunits: subunit A encompasses the catalytic domain responsible for ADP-ribosylation of elongation factor 2, which blocks protein synthesis of target cells, and subunit B is responsible for binding to the cell surface receptors and transferring subunit A into the cytoplasm (28). Immunity against diphtheria is obtained by the induction of a neutralizing Th2-dominant (mainly immunoglobulin G1 [IgG1]) humoral immune response against DTx. The conventional vaccine consists of the alum-adsorbed, formaldehyde-treated toxoid (diphtheria toxoid), administered to children in three doses at 1, 3, and 5 months, followed by boosters at 1.5 and 5 years of age. CRM<sub>197</sub> (cross-reacting material), a mutant DTx devoid of toxic activity, carries a unique glycine-to-glutamic acid substitution at...
residue 52 within the catalytic domain, which eliminates its toxic activity (8). It is used in several systems as the protein carrier for conjugated polysaccharide vaccines (15, 24). Native CRM₁₉₇ induces lower antibody levels than diphtheria toxoid, but its immunogenicity is improved after a mild formaldehyde treatment (12).

Expression and purification of recombinant CRM₁₉₇ in E. coli has been described (3). Expression of this antigen or its fragments in the recombinant Salmonella enterica serovar Typhi CVD 908-Δitet vaccine strain has proved to be compromised by the insolubility of the heterologous proteins (22). Solubilization by using the hemolysin A secretion system from E. coli resulted in low expression levels, and all constructs failed to induce immune responses. Recently, a Staphylococcus carnosus strain expressing the receptor-binding domain of DTx was shown to induce neutralizing antibodies after nine doses of 3 × 10⁸ CFU (7).

In this study, we analyzed the potential of CRM₁₉₇, as the antigen in an rBCG vaccine against diphtheria, with the long-term goal of developing an rBCG DPT vaccine. Here we describe the successful expression of CRM₁₉₇ in rBCG using E. coli/mycobacterium vectors, under the control of the pBlaF₈ promoter, an unregulated β-lactamase promoter isolated from Mycobacterium fortuitum. We also describe efficient priming of the DTx-neutralizing humoral response in mice immunized with rBCG-CRM₁₉₇.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and vaccine preparation. All cloning steps were performed in E. coli DH5α grown in Luria-Bertani medium supplemented with ampicillin (100 μg/ml) or kanamycin (20 μg/ml). The BCG Moreau strain was used to generate the rBCG strains. Liquid cultures of the BCG strains were regularly grown in Middlebrook 7H9 medium supplemented with albumin-dextrose-catalase (ADC; Difco, Detroit, Mich.), with or without kanamycin (20 μg/ml), at 37°C using stationary tissue culture flasks. The rBCG strains were cultured in Ungar’s medium (16) for the heterologous protein localization assays. BCG was transformed by electroporation as previously described (29) and plated onto Middlebrook 7H10 agar plates supplemented with oleic acid-ADC (Difco) containing kanamycin (20 μg/ml). Plates were Incubated at 37°C for 3 weeks before expansion of the transformed colonies in liquid media. rBCG vaccines were prepared from mid-log-phase liquid cultures of selected clones. The liquid cultures were centrifuged at 4,000 g, resuspended in 10% glycerol, and maintained at −80°C until used. The numbers of CFU in the frozen stocks were previously determined by growing the thawed vaccine preparations on Middlebrook 7H10 plates containing kanamycin (20 μg/ml) at 37°C. The supernatant was subjected to detergent preparation. The protein concentration in the culture supernatant was determined as described previously (22).

Constructions of the CRM₁₉₇ expression vectors. pJEM17, pLA71, and pLA73 contain the E. coli and mycobacterium origins of replication, a kanamycin resistance gene, the pBlaF₈ promoter, its ATG initiation codon, and a multicloning site (19, 27). pJEM17 expresses the native inserted gene, and pLA71 and pLA73 place the heterologous gene in fusion with either the β-lactamase signal sequence or the whole β-lactamase-encoding gene (Fig. 1). For the construction of pEL17CRM₁₉₇, the CRM₁₉₇ gene was PCR amplified from pMSM38, a Bacillus subtilis plasmid, without its signal sequence using the primers 5′-TAG TAG GGA TCC TAG CGC TGA TGA TGT TGA T3′ and 5′-TAG TAG GGA TCC GAT ATC TCA GCT TTT GAT TTC AAA TAG C3′. Underlining and italics indicate BamHI and EcoRV restriction sites, respectively. The amplified fragment (1,604 bp) was digested with BamHI and subcloned into pBCSK+ (Stratagene, La Jolla, Calif.). pBamHI/EcoRV fragment was further cloned into pEL17ΔTET. For construction of pEL71CRM₁₉₇, pEL73CRM₁₉₇, and pEL74CRM₁₉₇, the CRM₁₉₇ gene without its signal sequence was amplified by PCR with the primers 5′-TAG TAG GGA TCC TAC GTA CGG CGG CTG ATG ATG TTG TTC AT3′ and 5′-TAG TAG GGA TCC GAC CGC GCC GCT CAG GCT TTT ATT TCA AAA AAT AGC 3′. Underlining, italics, and bold type indicate BamHI, NotI, and subcloned into pEL71ΔTET and pEL73ΔTET, resulting in pEL71CRM₁₉₇ and pEL73CRM₁₉₇, which have the CRM₁₉₇ gene inserted in frame with β-lactamase fragment sequences.

Western blotting. Kanamycin-resistant BCG clones were grown in 50-ml Middlebrook 7H9-ADC liquid cultures supplemented with kanamycin (20 μg/ml). Cells from 25 ml of these cultures were harvested at mid-log phase by centrifugation, washed once with 5 ml of Tris-EDTA, resuspended in 0.5 ml of Tris-EDTA, and disrupted on ice for 2 min using a GE 100 ultrasonic processor at half-maximum constant output. The protein concentration in the culture lysates was determined with a protein assay (Bio-Rad, Hercules, Calif.), using bovine serum albumin (BSA) as a standard. Approximately 50-μg aliquots of protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) gel. The proteins were then electrotransferred onto a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H.), and the membrane was saturated with 5% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (vol/vol) (Sigma, St. Louis, Mo.) (PBS-T). Horse anti-diphtheria toxoid serum, routinely produced by Instituto Butantan (São Paulo, São Paulo, Brazil), was adsorbed against BCG to remove antibodies against mycobacterial antigens according to the method described by Gruber and Zingales (10) and used for detection of CRM₁₉₇ or diphtheria toxoid in the immunoblots (1:1,000). Horseradish peroxidase (HRP)-conjugated anti-horse antibody (Sigma) was used as secondary antibody, and detection was performed with an ECL kit (Amersham-Pharmacia, Little Chalfont, Buckinghamshire, England).

Localization of heterologous proteins in rBCG. Clones of the rBCG strains expressing the heterologous protein were grown in 30-ml cultures of Ungar’s medium supplemented with kanamycin (20 μg/ml). The cells were harvested at mid-log phase by centrifugation. The proteins from the culture supernatants were precipitated with acetone. The cell pellet was resuspended in PBS, with adjustment of cell density to equivalent values, and sonicated for 2 min as described above. Membranes were solubilized by the addition of 2% (vol/vol) Triton X-114. Insoluble material (cell wall-enriched fractions) were separated by centrifugation at 27,000 × g, and the supernatant was subjected to detergent phase partitioning, separating the membrane and cytosol fractions, as described elsewhere (26). Samples from each fraction were subjected to SDS-PAGE and immunoblotting as described above.

Immunizations. Male 4-week-old BALB/c mice from Instituto Butantan were immunized intraperitoneally (i.p.) with 10⁵ CFU of BCG, rBCG-CRM₁₉₇, or a mixture of 5 × 10⁶ CFU of rBCG-CRM₁₉₇ and 5 × 10⁵ CFU of rBCG-FC (rBCG expressing tetanus toxin fragment C) (Mazzantini et al., unpublished data) in 500 μl of apyrogenic saline. The conventional DT vaccine (1 Lf [limit of flocculation] of alum-adsorbed diphtheria toxoid and 0.25 Lf of tetanus toxoid per mouse) produced by Instituto Butantan was used as a positive control. Blood was collected from the retro-orbital plexus, and pooled sera were analyzed by enzyme-linked immunosorbent assay (ELISA) for antibodies against diphteria toxoid.

ELISA. Serum antibody responses to rBCG immunizations and controls were quantified by ELISA. Briefly, Polysorp 96-well plates (Nunc International, Rochester, N.Y.) were coated with diphtheria toxoid (Instituto Butantan) (100 μl; 2 μg/ml in carbonate-bicarbonate buffer, pH 9.6; 4°C overnight), washed three times with PBS-T, blocked with 10% nonfat dry milk in PBS, and then incubated with serial dilutions of mouse sera in PBS-1% BSA at 37°C for 1 h. The plates were washed as described above and incubated with HRP-conjugated goat anti-mouse IgG (1:2,000) (Sigma) in PBS-1% BSA at 37°C for 1 h. Antibody isotyp-
ing was performed using goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA (1:2,000) (Sigma) and HRP-conjugated anti-goat (1:10,000) antibodies (Sigma). Following washing, antibodies were visualized by adding OPD substrate (100 μl, 0.04% o-phenylenediamine in citrate phosphate buffer [pH 5], containing 0.05% H2O2). After color development (15 min), the reaction was interrupted by addition of 8 M H2SO4 (50 μl), and the A492 was determined. Absorbance values were plotted against serum dilutions.

RESULTS

Expression and localization of CRM197 in rBCG. We have constructed a series of plasmid vectors for expression of CRM197 in BCG under the control of the pBlacF promoter, either directly at the multicloning site in pEL17CRM197, in fusion with the β-lactamase signal sequence in pEL71CRM197, or with the whole β-lactamase sequence in pEL73CRM197 (Fig. 1). BCG was transformed with each of the three constructs, generating rBCG(pEL17CRM197), rBCG(pEL71CRM197), and rBCG(pEL73CRM197), respectively. Expression of the antigen in the different strains was analyzed by immunoblotting. Figure 2 shows that CRM197 was expressed in significant amounts by all constructs, but mainly bands with the predicted size for CRM197 (58 kDa) and bands possibly originating from proteolysis of the former one were detected. These results indicated that the protein fusions in rBCG(pEL71CRM197) and rBCG(pEL73CRM197) were not stable and were probably cleaved near the fusion point between the β-lactamase fragments and CRM197. Total cell extracts were further subjected to cellular fractionation to determine the localization of CRM197 in the BCG strains through immunoblotting. Surprisingly, in all the rBCG-CRM197 strains, CRM197 was mostly localized to the detergent-insoluble fraction, which is enriched in cell wall components (data not shown), even though the pEL17CRM197 construct does not carry any signal sequence for protein export.

Immune response to rBCG expressing CRM197. Eight BALB/c mice were immunized i.p. with 107 CFU of rBCG(pEL17CRM197), using rBCG(pJEM17) as a negative control, and given boosters under the same conditions after 9 weeks. An initial increase in anti-diphtheria toxoid antibody level was observed in the sera of mice immunized with rBCG(pEL17CRM197), and this level decreased to control levels in the following months (Fig. 3A). A more important increase was observed 2 months after the booster dose. Comparable results were obtained in mice immunized with rBCG(pEL71CRM197), since expression and localization of CRM197 were similar in all constructs (results not shown). At 20 weeks after priming (S5), the isotype profile of the sera of rBCG(pEL17CRM197)-immunized mice showed mainly IgG1 induction (Fig. 3B); IgG1 is considered the main antibody isotype responsible for DTx neutralization. Despite showing the expected isotype profile, these sera failed to neutralize DTx in the in vitro Vero cell neutralization assay.

Induction of DTx-neutralizing antibodies after rBCG priming. In order to determine if mice immunized with rBCG...
munized mice, showed that rBCG(pEL17CRM197) induced

ments. Isotyping of the antibodies present in the sera of im-

DT vaccine. Similar results were obtained in different experi-

reaching around 70% of that obtained with the conventional

antibody levels was observed after the subimmunizing dose,

of the conventional DT vaccine at 3 weeks after

were not found in the literature.

BlaF* promoter in fusion

p

lactamase or not fused. Strong

and BCG(pRL17FC) (rBCG expressing tetanus toxin frag-

were localized in the fraction enriched in bacterial

cell wall, even when no export signal sequences were present.

the intrinsic ability of DTx to directly interact with the lipid

membrane (4, 20) could be responsible for this unexpected

result. Furthermore, CRM197 was shown to bind more strongly

to the lipid bilayer than DTx (23). Alternatively, CRM197 could

be localized in inclusion bodies, which would precipitate to-

gether with cell wall components in the fractionation experi-

ments. Since entrapment of recombinant proteins in inclusion

bodies is normally associated with very high expression levels,

we consider the latter possibility unlikely, because the expres-

sion of CRM197 is not very high (as analyzed by SDS-PAGE

and Coomassie blue staining; results not shown). Furthermore,

elements of recombinant antigens localized in inclusion bodies

in rBCG were not found in the literature.

Immunization of mice with rBCG expressing the native

CRM197 gene in the rBCG(pEL17CRM197) strain followed

by a booster dose at 2 months under the same conditions

was able to elicit more important antibody responses only at

long intervals after priming (Fig. 3). At this point, rBCG

(pEL17CRM197) induced mainly IgG1 (Th2 response) anti-
diptheria toxoid antibodies, and this is the antibody isotype

considered responsible for the neutralization of DTx. How-

ever, the antibody levels induced in the sera of immunized

mice were insufficient to neutralize toxin activity, or the anti-
bodies could have been of a nonneutralizing type.

It has been observed that mycobacteria of the M. tuberculosis

complex (to which BCG belongs) induce strong cellular re-
sponses soon after infection, but humoral responses appear

late during the development of the disease. Immunizations

with rBCG expressing bacterial antigens are usually followed

during 4 to 6 months (5, 18, 26). We also observed a gradual

increase in the humoral response up to 5 months after priming.

On the other hand, it has been proposed that rBCG could elicit

a priming effect, which may enable the induction of a memory

response triggered by an infection (9). We thus analyzed the

effect of a subimmunizing dose of the conventional DT vaccine

at shorter intervals after rBCG priming, in an effort to mimic

an infection. A similar strategy has been used in mice immu-
nized with rBCG expressing FC (5). Indeed, mice immunized with rBCG (pRL17CRM$_{197}$) or its combination with rBCG (pRL17FC), induced a strong humoral response 3 weeks after a subimmunizing dose of DT (7 weeks after priming) (Fig. 4A). Interestingly, the induction of an IgG1-predominant (Th1 response) and neutralizing-antibody response was achieved only with the combination of rBCG strains expressing the diphtheria and tetanus antigens. These results indicate an adjuvant effect of rBCG-FC on the immune response induced by rBCG-CRM$_{197}$. It was recently shown that rBCG expressing E. coli heat labile enterotoxin (LT-Bh) induced a primary response shifted towards IgG2a, characteristic of the Th1 response typically associated with mycobacterial infections (14). We could detect a Th2-dominant response (mainly IgG1 antibodies) at long intervals after priming with rBCG-CRM$_{197}$ (Fig. 3B) or within a shorter time in animals injected with a subimmunizing dose of the conventional DT vaccine after priming with a mixture of rBCG-CRM$_{197}$ and rBCG-FC (Fig. 4B). These results might indicate that the antigen is more driving in the induction of the immune response, with the detection of a Th1-dominant response for LT-Bh and a Th2-dominant response for CRM$_{197}$.

Priming with a combination of rBCG strains expressing the diphtheria and tetanus antigens, followed by a subimmunizing dose of DT, induced an antibody response with neutralizing activity against DTx (0.16 IU/ml), as did the conventional DT vaccine (2.56 IU/ml), although at lower levels. Quality control of diphtheria vaccines is normally performed with sera from immunized guinea pigs, and the level of DTx neutralization required for vaccine certification is 0.5 IU/ml after a single dose. The diphtheria vaccine produced by Instituto Butantan consistently shows induction of neutralizing activity well above the minimum requirements (results not shown). Gupta and collaborators (12) described substantial differences between in vitro neutralization tests performed with sera from mice and guinea pigs immunized with CRM$_{197}$, showing significantly lower titers for mouse sera. Since our results were obtained with mice, the antibody levels induced by rBCG priming might be close to that required for approval of the conventional vaccines against diphtheria.

The expression of CRM$_{197}$ in rBCG was investigated with the aim of developing an rBCG DPT vaccine. The expression of FC has been achieved by several groups using different mycobacterial vectors, showing the induction of a neutralizing humoral response (2, 5). rBCG expressing the S1 subunit of pertussis toxin (PT) in fusion with FC has been shown to induce a specific T-cell response against PT, as well as a tetanus toxin-neutralizing humoral response (2). Furthermore, we have recently shown that rBCG expressing the genetically detoxified S1 subunit of PT-9K/129G in fusion with the β-lactamase signal sequence induces a cellular response which protects mice against an intracerebral challenge with live Bordetella pertussis (21). We now demonstrate that rBCG expressing CRM$_{197}$ can induce a neutralizing humoral response against DTx. Taken together, these results further encourage studies on the development of a one-dose rBCG vaccine eliciting protective immunity against diphtheria, pertussis, and tetanus. Enhancement of the immune response elicited by rBCG-CRM$_{197}$ will be investigated and could perhaps be achieved when the vaccine is administered in combination with rBCG expressing tetanus and pertussis antigens. The administration of rBCG vaccines to humans would also require improvements, such as the elimination of antibiotic resistance markers and stable expression of the antigens through insertion of sequences into the mycobacterial genome, goals that are currently being pursued in our laboratory.

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