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Recombinant *Mycobacterium bovis* BCG Secreting Functional Interleukin-2 Enhances Gamma Interferon Production by Splenocytes

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***Mycobacterium bovis* BCG was genetically engineered to express and secrete mouse interleukin-2 (IL-2) and rat IL-2. Genes encoding IL-2 were inserted into an *Escherichia coli*-BCG shuttle plasmid under the control of the BCG HSP60 promoter. To facilitate study of proteins produced in this system, the IL-2 gene product was expressed (i) alone, (ii) with the mycobacterial alpha-antigen secretion signal sequence at the amino terminus, (iii) with an influenza virus hemagglutinin epitope tag at the amino terminus, and (iv) with both the secretion signal sequence and the epitope tag. When expressed with the alpha-antigen signal sequence, biologically active IL-2 was secreted into the extracellular medium. Western blot (immunoblot) analysis of the intracellular IL-2 and extracellular IL-2 revealed that the secretion signal was appropriately cleaved from the recombinant lymphokine upon secretion. To assess the ability of recombinant BCG to stimulate cytokine production in a splenocyte population, mouse splenocytes were cultured together with wild-type or IL-2-producing BCG. IL-2-secreting BCG clones stimulated substantial increases in gamma interferon production, which could be reproduced by the addition of exogenous IL-2 to BCG. Levels of IL-6, IL-10, tumor necrosis factor alpha, and granulocyte-macrophage colony-stimulating factor were not significantly changed, while IL-4 and IL-5 remained undetectable (less than 50 pg/ml). The enhanced production of gamma interferon in response to IL-2-secreting BCG was strain independent. Recombinant BCG expressing mammalian cytokines provides a novel means to deliver cytokines and may augment the immunostimulatory properties of BCG in immunization and cancer therapy.**

Mycobacterium bovis BCG (bacillus Calmette-Guérin) (4) is an important clinical tool because of its profound immunostimulatory properties. Cell wall extracts of BCG have proven to have excellent immune adjuvant activity (6, 44). Live BCG is an effective and safe vaccine used worldwide to prevent tuberculosis (25, 26). Live BCG has also been successfully used clinically as an antitumor agent in the treatment of both superficial bladder cancer and malignant melanoma (24, 28, 32, 33). Despite this widespread use of BCG, the mechanisms responsible for its diverse effects are still poorly understood.

Studies with cultured cells in vitro have documented that BCG is able to stimulate the expression of cytokines interleukins 1 to 4 and 6 (IL-1 to -4 and -6), tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) from mononuclear cells (17, 40, 49). Animal and human studies have confirmed these findings and demonstrated that BCG inoculation can activate various cellular compartments in vivo, including $CD4^+$ and $CD8^+$ T cells, γδ T cells, B cells, macrophages, and NK cells (3, 17, 18, 22, 27, 36, 37, 40, 51, 54–56). The cellular response to BCG exposure is complex, and the pattern of cytokine production is dependent on the genotype of mice used in these studies (17).

While the complex immunologic stimulation engendered by BCG probably contributes to its efficacy as a vaccine, this complexity has also made it difficult to identify the mechanisms responsible for BCG's clinical effects. An understanding of BCG's mechanism of action might suggest ways to improve BCG's clinical efficacy. With the advent of recombinant techniques for mycobacteria (1, 16, 19, 30, 43, 45), it should be possible to genetically alter BCG's immunostimulatory properties by the inclusion of genes encoding immunomodulatory molecules in the BCG genome. Thus, genetic modifications might enhance BCG effectiveness and could provide clues to the mechanisms involved in BCG's clinical properties.

An important feature of the cellular immune response to mycobacterial infection involves the recruitment of specific subsets of T helper cells (2, 12, 48). TH-1 cells, a primary source of IFN-γ and IL-2, mediate T cytotoxic activity and delayed-type hypersensitivity. TH-2 cells provide B-cell help for specific antibody isotype production and are characterized by the elaboration of the cytokines IL-4, -5, and -10 (8, 35). The specific type of T helper response has been shown to be influenced by the cytokine environment at the site of antigen presentation (11) and the genetic background (15, 17). Furthermore, distinct pathology has been associated with each of these specific cellular responses (52). Thus, it may be possible to direct the immune response toward that which is most beneficial in protection against specific pathogens (15). Similarly, the use of specific cytokines might reset an unproductive or deleterious cellular immune response (21).

In this article, we describe the genetic engineering of BCG to effectively express and secrete mammalian cytokines. Using IL-2 as the prototype cytokine, a fusion protein containing a BCG-specific signal peptide and an influenza virus hemagglutinin epitope tag was constructed, allowing the production, secretion, and detection of biologically active recombinant

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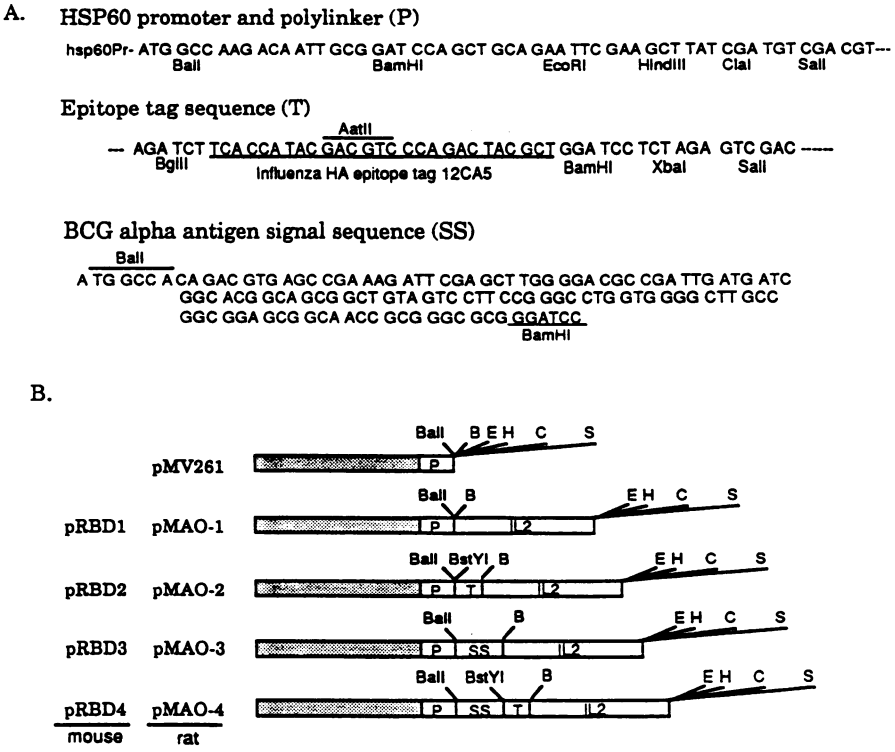

FIG. 1. Construction of recombinant expression vectors. (A) DNA sequences of relevant portions of pMV261 HSP60 promoter and polylinker, influenza virus hemagglutinin (HA) epitope tag sequence, and BCG alpha-antigen signal sequence used in construction of IL-2-containing *E. coli*-BCG shuttle plasmids. (B) Schematic illustration of IL-2-containing plasmid constructs with restriction sites. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; C, *Cla*I; S, *Sal*I.

IL-2 from BCG. We found that the BCG recombinants secreting IL-2 caused enhanced IFN-γ production by mouse splenocytes in vitro that was mouse strain independent.

MATERIALS AND METHODS

Oligonucleotide primers, plasmid DNAs, and bacterial strains. Three sets of paired oligonucleotide primers were utilized in the PCR with appropriate templates to produce insert DNAs with ends suitable for cloning in plasmid pMV261 (45). The oligonucleotide primers were GGCATGGC CAAGGGATCCGCACCCACTTCAAGCCCTGCA (primer 1) and CGGAATTCTTACTGAGTCATTGTTGAGATGAT (primer 2) (for the rat IL-2 gene), CAAGGGATCCGCAC CCACTTCAAGCCCTGCA (primer 3) and GCCGGAAT TCTTACTGAGTCATTGTTGAGATGAT (primer 4) (for the mouse IL-2 gene), and GCCATGCCACAGACGTGAGC CGAAAGATTCGA (primer 5) and GCCGGGATCCC GCGCCCGCGGTTGCCGCTCCGCC (primer 6) (for the alpha-antigen signal sequence [29]). The rat and mouse IL-2 upstream primers (primers 1 and 3, respectively) were constructed to anneal with the IL-2 coding regions starting at codon 21, thereby excluding their native signal peptide regions. The BCG alpha-antigen downstream primer 6 terminated at the sequence encoding the putative protease cleavage site Ala-Gly-Ala of the BCG signal peptide (46, 47) (Fig. 1A).

The rat IL-2 cDNA-containing plasmid pRIL-2.8 was provided by A. McKnight, and the mouse IL-2 cDNA plasmid pmut-1 was obtained through the American Type Culture Collection (31, 53). The *Escherichia coli*-BCG shuttle plasmid pMV261 was kindly provided by C. K. Stover (45). The influenza virus hemagglutinin epitope tag sequence is described in references 23 and 56 and had been cloned in the *Bgl*II and *Bam*HI sites of pSP72 (Promega).

E. coli MBM 7070 was obtained as a gift from John Seidman. *M. bovis* BCG (Pasteur) obtained from the American Type Culture Collection was grown in 7H9 Middlebrook medium containing 10% albumin dextrose solution (Difco) and 0.05% Tween 80 (Sigma). Genomic BCG DNA was isolated by protease K digestion and phenol-chloroform extraction.

Construction of IL-2 expression vectors and BCG IL-2 recombinant strains. A schematic representation of the plasmids constructed for this study is given in Fig. 1B. Plasmid pMAO-1 was constructed by placing the appropriate *Bal*I-*Eco*RI-digested rat IL-2 PCR insert into the similarly restricted parental plasmid, pMV261 (Fig. 1A). Plasmid pMAO-2 was obtained by first cloning the *Bam*HI-*Sal*I insert from pMAO-1 into the hemagglutinin epitope tag sequence-containing plasmid (Fig. 1A) and then placing the resulting *Bgl*II-*Eco*RI insert into the *Bam*HI-*Eco*RI site of pMV261. Plasmid pMAO-3 was constructed by cloning the *Bal*I-*Bam*HI-restricted PCR product encoding the alpha-antigen signal sequence into the *Bal*I-*Bam*HI site of pMAO-1. Plasmid pMAO-4 was produced by replacing the *Bam*HI-*Eco*RI insert of pMAO-3 with the same *Bgl*II-*Eco*RI insert used in preparing pMAO-2. A similar set of mouse IL-2-containing plasmids, pRBD-1 to -4, was produced by replacing the *Bam*HI-*Eco*RI rat cDNA insert in each of the respective pMAO plasmids with the PCR-derived *Bam*HI-*Eco*RI-flanked mouse IL-2 cDNA fragment. All DNA manipulations followed previously described procedures (39). *E. coli* MBM 7070 was electroporated with the IL-2-containing BCG-*E. coli* shuttle plasmids and

TABLE 1. BCG plasmids

Plasmid	Relevant characteristic(s)[a]
pMAO1	Rat IL-2 gene
pMAO2	Epitope-tagged rat IL-2 gene
pMAO3	Alpha-antigen signal sequence + rat IL-2 gene
pMAO4	Alpha-antigen signal sequence + epitope-tagged rat IL-2 gene
pRBD1	Mouse IL-2 gene
pRBD2	Epitope-tagged mouse IL-2 gene
pRBD3	Alpha-antigen signal sequence + mouse IL-2 gene
pRBD4	Alpha-antigen signal sequence + epitope-tagged mouse IL-2 gene

[a] Cloned into pMV261, an *E. coli*-BCG shuttle plasmid containing the HSP60 promoter.

selected on kanamycin (30 μg/ml) Luria-Bertani agar plates. The correct plasmid structures were confirmed on the basis of restriction analysis, DNA sequencing, and production of functional IL-2 (see below). *E. coli*-derived plasmids were then used to transform BCG by electroporation according to published procedures (43). BCG colony DNAs were individually tested by PCR for the presence of the IL-2 gene, and colony lysates were assayed for expression of functional IL-2 (see below).

Detection of recombinant IL-2. The expression of recombinant IL-2 in BCG was examined by Western blot (immunoblot) and bioassay. Sonicated BCG lysates from equivalent-density log-phase cultures and BCG culture medium from stationary-phase cultures were electrophoresed on a 17 to 27% acrylamide gel (Daiichi) and transferred to nitrocellulose. After being blocked with a 15% solution of powdered skim milk, the membrane was incubated overnight with the primary antibody, either rabbit anti-mouse IL-2 (Collaborative Research) or mouse monoclonal anti-hemagglutinin epitope tag sequence antibody 12CA5, at a concentration of 1 μg/ml (50). Peroxidase-labeled goat anti-rabbit or goat anti-mouse immunoglobulin G antibodies (Pierce) were used with a chemiluminescent substrate (Amersham) to detect membrane-bound IL-2.

The presence of biologically active IL-2 in bacterial extracts or conditioned media was determined and quantified colorimetrically in a proliferation assay using the IL-2-dependent T-cell line CTLL-2 (34). Maximal signals generated in this assay were similar for mouse IL-2 and rat IL-2. *E. coli* and BCG lysates were obtained by sonication of washed bacterial cells in phosphate-buffered saline (PBS) at 4°C followed by filtration through a 0.22-μm-pore-size filter and dialysis against PBS. No IL-2 inhibitors were found when CTLL-2 cells were incubated with exogenous IL-2 in the presence of extracts prepared from bacteria transformed with the nonproducer plasmid pMV261. To standardize the collection of BCG bacteria and conditioned media, log-phase BCG cultures were washed and resuspended at an optical density at 600 nm of 0.5 in fresh media containing 0.05% Tween 80 to prevent bacterial clumping. BCG clones expressing the IL-2 gene did not differ significantly in their growth rate, as measured either by optical density or by colony count, from each other or from BCG clones containing the same plasmid lacking the IL-2 gene (pMV261). At the end of 48 h, the optical density at 600 nm was readjusted to 1.0 by diluting the BCG cells with fresh media. The amount of IL-2 in 1 ml (1.0 U of optical density at 600 nm ~ 2.5×10^7 CFU) of cleared conditioned media, or in the pellet derived from 1 ml of cells, was then assessed in the proliferation assay.

In vitro spleen cell assay for cytokine production. Spleens were harvested from 8- to 12-week-old female C3H/HeN, C57BL/6, or BALB/c mice (Charles River). After mechanical dispersion, a mononuclear suspension of the spleen cells from pooled groups of 3 to 5 mice per strain was prepared by Ficoll-Paque (density, 1.077 g/liter; Pharmacia) centrifugation at 500 × *g*, washed, and placed into RPMI 1640 medium supplemented with HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 10% heat-inactivated fetal bovine serum, and 30 μg of kanamycin per ml. Splenocyte assays were performed in triplicate at a cell density of 2×10^6 cells per well in 24-well tissue culture plates (Nunc) in the presence or absence of exogenous murine recombinant IL-2 (Biosource) and 2×10^6 CFU of either MV261 BCG (henceforth referred to as wild-type BCG) or IL-2 recombinant BCG. Medium was removed at 24 and 72 h, centrifuged, and frozen at −70°C until testing in enzyme-linked immunosorbent assays (ELISAs). Equal spleen cell counts and viabilities were verified prior to final harvest by trypan blue counting. Parallel wells containing different recombinant BCG clones in tissue culture media supplemented with 0.05% Tween 80 demonstrated similar changes in optical density for each day of the experiment. Cytokine production by spleen cells was measured by sandwich ELISAs using paired monoclonal antibodies according to the manufacturer's instructions (Pharmingen). Epitope-tagged recombinant mouse IL-2 was captured in wells precoated with rabbit anti-mouse IL-2 and detected by using the murine monoclonal antibody 12CA5 at 1 μg/ml and alkaline phosphatase-labeled goat anti-mouse immunoglobulin G (Pierce).

RESULTS

Construction of BCG recombinants producing IL-2. A variety of *E. coli*-BCG shuttle plasmids were constructed to permit production of IL-2 (Fig. 1 and Table 1). A set of plasmids (pRBD-1 and pMAO-1) in which the BCG HSP60 promoter drives the expression of mouse or rat IL-2 was constructed. To permit differentiation of the BCG-produced recombinant IL-2 from IL-2 produced by mammalian cells in later experiments, a second set of plasmids (pRBD-2 and pMAO-2), which incorporated an influenza virus hemagglutinin epitope-coding sequence at the 5′ end of the IL-2 coding sequence to produce an epitope-tagged IL-2 molecule, was generated. To allow secretion of the recombinant IL-2 molecules, the secretion signal sequence of the mycobacterial alpha antigen was added to the 5′ end of the IL-2-coding sequence in a third set of plasmids (pRBD-3 and pMAO-3). A fourth set of plasmids (pRBD-4 and pMAO-4) contained both the epitope tag and the secretion signal sequence upstream of IL-2. All constructs containing the IL-2 gene were found to produce biologically active IL-2 in *E. coli* (data not shown).

BCG cells were transformed with all of the recombinant plasmids. The BCG transformation efficiencies for both the parental pMV261 plasmid and the constructs containing the alpha-antigen signal sequence were on the order of 10 to 100

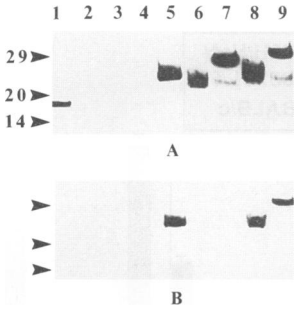

FIG. 2. Expression of IL-2 in Western blots of BCG pellet lysates (PL) and conditioned media (CM) from murine IL-2 BCG recombinants. Lane 1, recombinant murine IL-2; lanes 2 and 3, MV261 CM and PL, respectively; lanes 4 and 5, RBD-2 CM and PL, respectively; lanes 6 and 7, RBD-3 CM and PL, respectively; lanes 8 and 9, RBD-4 CM and PL, respectively. (A) Polyclonal rabbit anti-murine IL-2 antibody; (B) murine monoclonal antibody 12 CA5, specific for the influenza virus epitope tag. Positions of molecular weight markers are indicated on the left (in thousands).

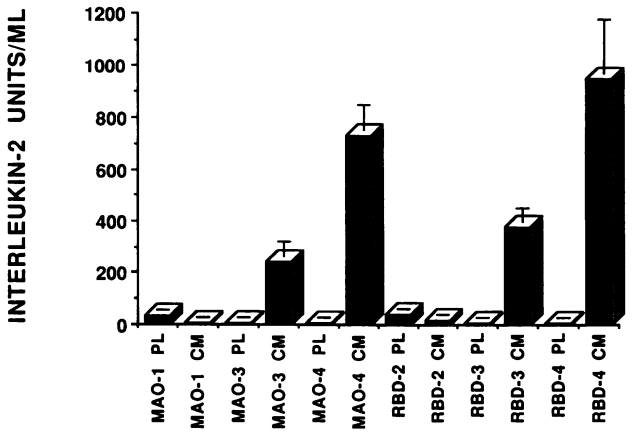

FIG. 3. Expression of biologically active IL-2 in BCG pellet lysates (PL) and conditioned media (CM) of rat (MAO) and mouse (RBD) IL-2 recombinant BCG clones. Values for MAO-3 and -4 and RBD-3 and -4 represent means of the three highest producer clones identified by ELISA screening. Only one clone each was available for MAO-1 and RBD-2.

times greater than for IL-2 constructs lacking the signal sequence. This was a uniform finding occurring in both mouse IL-2- and rat IL-2-containing constructs and may be due to a selective disadvantage caused by the intracellular accumulation of this foreign protein.

IL-2 production and secretion by BCG transformants. The expression of IL-2 protein by representative BCG recombinants was assayed by probing Western blots with antibodies directed against murine IL-2 (Fig. 2A) or against the influenza virus hemagglutinin epitope (Fig. 2B). BCG recombinants that expressed IL-2 without a secretion signal sequence accumulated a single form of IL-2 intracellularly (Fig. 2A, lane 5) but no IL-2 extracellularly (Fig. 2A, lane 4). High- and low-molecular-weight forms of IL-2 accumulated in BCG recombinants that expressed IL-2 linked to the secretion signal (Fig. 2A, lanes 7 and 9); only the lower-molecular-weight form was found in the culture medium, an observation consistent with the cleavage of the signal sequence during secretion (Fig. 2A, lanes 6 and 8). The recombinant IL-2 proteins that contained the influenza virus hemagglutinin epitope tag were also visualized with a monoclonal antibody specific for the tag (Fig. 2B, lanes 5, 8, and 9).

The expression of functional IL-2 protein by representative BCG recombinants was investigated by an IL-2-dependent proliferation assay (Fig. 3). Most of the biologically active IL-2 produced by clones MAO-1 and RBD-2 was located in the pellet, while most of the IL-2 product from clones MAO-3, MAO-4, RBD-3, and RBD-4 was found in the extracellular medium. BCG clones expressing IL-2 linked to the alpha-antigen signal peptide (MAO-3, MAO-4, RBD-3, and RBD-4) produced significantly more biologically active IL-2 than clones without the signal peptide (MAO-1 and RBD-2). The reason for the apparent enhancement of IL-2 secretion associated with recombinant BCGs MAO-4 and RBD-4 containing the influenza virus epitope tag is unknown but may relate to the additional physical separation provided by the tag peptide sandwiched between the signal peptide and IL-2 domains. Both mouse and rat IL-2 BCG recombinants expressed similar amounts of biologically active IL-2. The amounts of recombinant mouse IL-2 in pellets and in media were also measured by an ELISA, and similar results were obtained (data not shown).

Stimulation of splenocyte cytokine production with BCG IL-2 recombinants. To evaluate the immunostimulatory properties of IL-2-secreting BCG, the abilities of BCG recombinants to alter the levels of cytokines IL-2 to -6 and -10, TNF-α, granulocyte-macrophage colony-stimulating factor, and IFN-γ produced by naive cultured murine spleen cells were investigated (Fig. 4). Splenocytes derived from BALB/c mice were incubated with either no BCG, 25 U of recombinant IL-2, MV261 BCG, nonsecretory IL-2 recombinant BCG (RBD-2), or secretory IL-2 recombinant BCG (RBD-3). Concanavalin A at a concentration of 10 μg/ml was used to demonstrate functional viability. The levels of specific cytokines in tissue culture media were measured by ELISA 72 h after the start of the experiment for all but TNF-α, which peaked at an earlier (24-h) time point.

The data show that no significant basal cytokine expression was detected from splenocytes in the absence of BCG or exogenous IL-2. In the IL-2-treated group, there was a modest elevation in IFN-γ production over the time course of the experiment but no detectable increase in other cytokines. The most significant cytokine production was observed with splenocytes exposed to BCG recombinants secreting IL-2. Substantially higher levels of IFN-γ were produced when spleen cells were exposed to recombinant IL-2-secreting BCG than when they were exposed to either wild-type BCG or recombinant IL-2-nonsecreting BCG. IL-2 production from spleen cells also appeared to increase slightly, although approximately 90% of detected total IL-2 was from recombinant BCG (the data were obtained by utilizing the tag of the recombinant IL-2 produced by RBD-4). Finally, there was a modest increase in granulocyte-macrophage colony-stimulating factor from spleen cells exposed to IL-2-secreting recombinant BCG. Splenocytes exposed to either wild-type BCG or recombinant BCG produced similar amounts of IL-6, IL-10, and TNF-α. We did not detect significant amounts of either IL-4 (Fig. 4) or IL-5 (data not shown) in these splenocyte cultures (lower assay limit, 50 pg/ml) under any of these experimental conditions.

Splenocyte cytokine stimulation by recombinant BCG occurs independently of mouse strain. There is a marked genetic variation in immune responses in different mouse strains after BCG infection (17). To determine whether the enhanced immunostimulatory properties of IL-2-secreting BCG were strain independent, splenocytes were isolated from three dif-

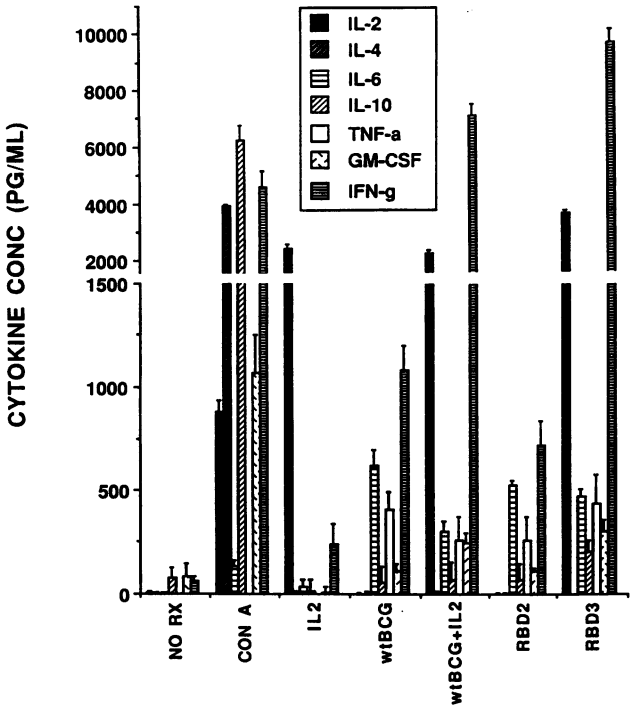

FIG. 4. Cytokine production in vitro by BALB/c splenocytes following incubation alone (NO RX), with 10 μg of conconavalin A (CON A) per ml, with 25 U (2500 pg/ml) of exogenous recombinant murine IL-2, with wild-type BCG (wtBCG), with recombinant IL-2-nonsecreting BCG (RBD2), or with recombinant IL-2-secreting BCG (RBD3). Values were obtained by ELISA of conditioned medium after 72 h of incubation for all cytokines except TNF-α, which was measured at 24 h. Values are expressed as means from triplicate well incubations, with error bars representing one standard deviation. Similar results were obtained in two other independent experiments. GM-CSF, granulocyte-macrophage colony-stimulating factor.

ferent mouse strains, C3H/HeN, C57BL/6, and BALB/c. The splenocytes were exposed to wild-type or recombinant BCG and assayed for specific cytokine secretion in tissue culture. The results for IFN-γ are shown in Fig. 5. In the absence of BCG or exogenous IL-2, there was minimal basal cytokine expression. IL-2 treatment or wild-type BCG treatment alone resulted in small elevations in IFN-γ production. Similar amounts of IL-6 and -10 and TNF-α were produced in response to either wild-type BCG or recombinant BCG. The expression of granulocyte-macrophage colony-stimulating factor was approximately twofold higher for BALB/c and C57BL/6 splenocytes in response to the combination of IL-2 plus BCG or IL-2-secreting recombinant BCG, but no difference was seen with C3H/HeN splenocytes. No IL-4 was produced in any strain as a result of exposure to BCG, IL-2, or the combination (data not shown).

In contrast, the exposure of splenocytes to BCG plus exogenously supplied IL-2 or to the BCG IL-2-secreting recombinant led to substantial increases in IFN-γ production with all three mouse strains studied. The tagged IL-2-secreting recombinant BCG produced responses similar to those of the nontagged secretor. Secretion of IL-2 appeared to be essential for the enhanced production of IFN-γ, as the nonsecreting BCG recombinant failed to elicit this response. The levels of IFN-γ produced by splenocytes showed IL-2 concentration dependence and saturability, and repeated experiments gave

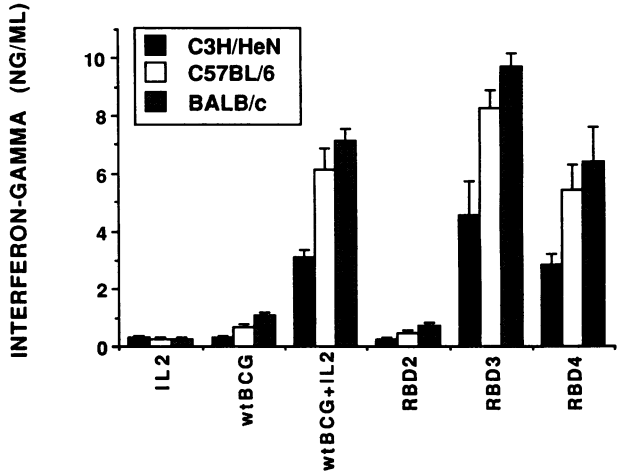

FIG. 5. Comparison of the levels of IFN-γ production from C3H/HeN, C57BL/6, and BALB/c splenocytes under conditions described for Fig. 4. Tagged secreted IL-2 is expressed by RBD4. wt, wild type.

comparable results (data not shown). These results indicate that the enhanced immunostimulatory properties of IL-2-secreting BCG are not strain dependent.

DISCUSSION

We have constructed *M. bovis* BCG recombinants that produce and secrete the mammalian cytokine IL-2 in a biologically active form. Secretion of the active cytokine was accomplished through the combined use of the BCG HSP60 promoter and a secretion signal sequence derived from the BCG alpha antigen. The BCG recombinants that secrete IL-2 produced a synergistic increase in IFN-γ in mouse splenocyte cultures substantially more than nonrecombinant BCG, demonstrating that BCG recombinants that express IL-2 and possibly other lymphokines can be used to modify the levels of specific cytokine production. These results indicate that recombinant BCG can perform as a functional cytokine delivery vehicle.

In principle, any cytokine gene can be introduced and expressed in BCG. The selection of the cytokine IL-2 as the first recombinant cytokine to be tested for secretion from BCG was based on the previously documented role of T-cell-mediated immune responses to mycobacterial infection (9). The modified *E. coli*-BCG shuttle vectors described here can be used to express and secrete any variety of cytokines. The expression of epitope-tagged cytokines from some of these vectors provides an additional assay for the presence of the cytokine. By being able to discriminate between the recombinant cytokine produced by BCG and the cytokine produced by mammalian cells, a more accurate picture of the mechanism of enhanced immunological stimulation can be obtained.

As a prototype cytokine expression system for BCG, we have demonstrated the extracellular accumulation of biologically active rat IL-2 and mouse IL-2 when the alpha-antigen signal sequence is fused with the IL-2 coding sequence. Additional evidence that the signal peptide was responsible for secretion was found in the Western blot analysis of the BCG recombinants. For each of the BCG recombinants that incorporated the signal sequence, the expressed IL-2 polypeptide appeared to accumulate inside BCG cells both with and without the signal peptide; in contrast, the size of the single secreted form

of IL-2 was consistent with that expected for IL-2 after the signal peptide has been cleaved. Matsuo et al. previously demonstrated that human immunodeficiency virus epitopes fused to the full-length alpha antigen from *Mycobacterium kansasii* were secreted with the modified protein after signal peptide cleavage (30). However, there are no previous reports that the BCG alpha-antigen signal peptide itself could direct the extracellular secretion of a full-length foreign protein from BCG.

We chose an in vitro model of immune stimulation using a mixed population of mononuclear cells derived from spleen cells to determine if IL-2-secreting BCG would stimulate cytokine production differently from wild-type BCG. Since specific cytokine production has been shown to be influenced by the genetic background of the host mouse strain, we examined the effects of recombinant BCG on the production of IFN-γ and other cytokines in three different mouse strains. Significant increases in IFN-γ production were observed when splenocytes from different mouse strains were exposed to IL-2-secreting recombinant BCG, despite the fact that different basal responses to wild-type BCG occurred among the mouse strains. This observation is especially interesting for BALB/c mice, in which susceptibility to BCG dissemination has been attributed to the strain's relatively poor IFN-γ response to intracellular infection (15).

Since no attempt was made to fractionate cellular subsets, the observed increase in IFN-γ could certainly have been of non-T-helper-cell origin such as NK cells, $CD8^+$ T cells, or even $CD4^-$ $CD8^-$ γδ T cells. Whatever the source of IFN-γ detected in our assay, the potential impact of a modified immune response to BCG associated with high local levels of IFN-γ and IL-2 may be significant if it is reproduced in vivo. IFN-γ has been shown to rescue BALB/c mice infected with specific intracellular organisms such as *Leishmania major* largely by affecting a TH-1 response (7, 15). Experiments with IFN-γ and IFN-γ receptor knockout mice have also substantiated the role of IFN-γ in eliminating mycobacterial infection (14, 20). Recombinant BCG secreting IL-2 and inducing IFN-γ in the local environment at the onset could potentially shift an uncommitted T-helper response in the direction of a TH-1-cell-mediated response (41, 42). Other live cytokine-expressing vectors, such as *Salmonella typhimurium* or vaccinia virus, have been shown to modify the native immune response to the vector (5, 13, 38).

With the advent of recombinant techniques for introduction of foreign antigens into mycobacteria, BCG has been proposed as a particularly useful vaccination vehicle (1, 10, 45). The modifications described here, whereby BCG is engineered to provide a source of biologically active cytokines, represent an additional and novel means to enhance the host immune response. Ultimately, such an approach may allow the clinical usefulness of this organism to be extended further.

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