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Manipulation and potentiation of antimycobacterial immunity using recombinant bacille Calmette–Guérin strains that secrete cytokines

PETER J. MURRAY*, ANNA ALDOVINI†, AND RICHARD A. YOUNG*‡

*Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142; †Department of Pediatrics, Children's Hospital and Harvard Medical School, Boston, MA 02115; and ‡Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

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ABSTRACT **Bacille Calmette–Guérin (BCG) is a live, attenuated strain of *Mycobacterium bovis* used widely for tuberculosis prophylaxis and bladder cancer immunotherapy, although it has limitations in both contexts. To investigate whether BCG's immunostimulatory properties could be modified, and to gain insight into the interaction between mycobacteria and their hosts, we constructed recombinant BCG strains that secrete functional murine cytokines and studied their properties in mouse models of experimental infection. Cell-mediated immune responses to mycobacterial antigen (purified protein derivative) were assayed using splenocytes from mice inoculated with various BCG recombinants. Antigen-specific proliferation and cytokine release were found to be substantially greater with splenocytes derived from mice injected with cytokine-secreting BCG than with splenocytes from mice injected with BCG lacking cytokines. The most profound effects were induced by BCG secreting interleukin 2, interferon γ, or granulocyte–macrophage colony-stimulating factor. Thus, cytokine-secreting BCG can enhance immune responses to mycobacterial antigens and may be improved reagents for tuberculosis prophylaxis and cancer immunotherapy.**

Among infectious diseases, tuberculosis (TB) remains the preeminent cause of death in most human societies. The World Health Organization estimates that the causative agent, *Mycobacterium tuberculosis*, has infected one-third of the human population (1). A small percentage of infected individuals develop clinical symptoms and succumb to the disease, accounting for the ≈3 million deaths per annum (2). In many cases, the host response to mycobacteria limits the proliferation of the pathogen but does not produce sterilizing immunity. Because of their impact as human pathogens and as a result of their profound immunostimulatory properties, mycobacteria have been widely used for basic immunological investigations and have become important tools for TB prophylaxis and cancer immunotherapy.

Early in this century, Calmette and his colleagues developed an attenuated strain of *Mycobacterium bovis*, bacille Calmette–Guérin (BCG), for use as a vaccine against TB (3). BCG is among the most widely used human vaccines, in part because it requires only a single inoculation, it can be given at birth, and with over 2 billion individuals vaccinated, it has a long record of safe use (4, 5). However, the variable efficacy of BCG against TB observed in different vaccine trials and the increase in drug-resistant TB cases worldwide suggest the need for an improved vaccine against TB.

The profound immunostimulatory properties of BCG have also been exploited in the therapy of certain human cancers, in particular, superficial bladder cancer (6, 7). Intravesical administration of BCG has a high efficacy against superficial bladder carcinomas, but large and frequent doses are required, occasionally leading to disseminated mycobacteriosis. Thus, there is a need for BCG strains that are effective at lower doses.

The ability to genetically manipulate mycobacteria (8, 9) allows us to explore the possibility that the inherent immunostimulatory properties of BCG could be modified or potentiated through the secretion of mammalian cytokines. We have investigated whether a broad spectrum of cytokines can be produced and secreted in active form by BCG and have studied whether cytokine secretion affects the immunological properties of BCG in mice. We report here that a surprisingly large fraction of murine cytokines tested thus far can be produced and secreted in active form by BCG and that certain cytokine-secreting BCG are substantially more potent stimulators of the cell-mediated immune response than their normal BCG counterparts.

MATERIALS AND METHODS

Construction of BCG Strains That Secrete Murine Cytokines. Murine cDNAs for interleukin (IL) 4, IL-6, granulocyte–macrophage colony-stimulating factor (GM-CSF), and interferon γ (IFN-γ) were cloned into the plasmids pRBD3 and pRBD4, which have been described (10), along with the BCG strain secreting IL-2. The expression vectors have a kanamycin-resistance gene for selection in both BCG and *Escherichia coli*, the *HSP60* promoter upstream of the insert, a polylinker for insertion of the cDNA, and DNA encoding the signal sequence derived from the BCG alpha antigen gene that targets the expressed protein for secretion through the mycobacterial cell membrane and wall (11). cDNAs were modified by PCR to replace the endogenous signal sequence-encoding DNA with the following oligonucleotides: GM-CSF (PM32, CGCGGATCCGCACCCACCCGCTCA; and PM33, GCGGAATTCTCATTTTTGGCTTGGTTT), IL-6 (PM34, CGCGGATCCTTCCCTACTTCACAA; and PM35, GCGGAATTCCTAGGTTTGCCGAGTAGA), IL-4 (PM36, CGCGGATCCAGGAGCCATATCCAC; and PM37, GCGGAATTCCTACGAGTAATCCATTTG), and IFN-γ (PM38, CGCGGATCCCACGGCACAGTCATT; and PM39, GCGGAATTCTCAGCAGCGACTCCTTTT). Fragments were digested with *Eco*RI and *Bam*HI and cloned into pRBD3 or pRBD4 similarly digested.

Transformation and Growth of Mycobacteria. BCG (Pasteur strain) was transformed using previously published procedures (8, 12). To identify BCG clones that contained the plasmid of interest, BCG from liquid cultures was spotted onto nitrocellulose and allowed to air dry. The filters were autoclaved for 2 min and treated in the standard manner for colony hybridizations. The filters were screened by hybridization with the relevant cytokine cDNA as a probe. Positive clones were

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Abbreviations: TB, tuberculosis; PPD, purified protein derivative; BCG, bacille Calmette–Guérin; IL, interleukin; IFN-γ, interferon γ; GM-CSF, granulocyte–macrophage colony-stimulating factor.

further screened by examining the supernatant for the relevant cytokine by ELISA and bioassay. The highest producing cultures (expressed as picograms of cytokine per milliliter per OD_{600}; see Table 1) were used in all subsequent experiments.

Cytokine Assays. Supernatants were taken from BCG cultures that had reached an approximate OD_{600} of 0.5–1.0; these were filtered through a 0.22-μm filter and stored at −70°C. Supernatants were screened by both ELISA and bioassay using standard procedures and cell lines. The ELISA kits used were supplied by Endogen (Cambridge, MA) (IL-2, IL-4, IL-6, GM-CSF, and IFN-γ) or Genzyme (IL-2 and IFN-γ) and were chosen for their high sensitivity in detecting cytokines in BCG culture supernatants. The factor-dependent cell lines used for bioassays were CTLL and HT2 (IL-2), CT.4S (IL-4), FDC-P1 (GM-CSF), 7TD1 and KD83 (IL-6), and WEHI 279 and MB49.1 (IFN-γ). The source of each line was: CTLL, CT.4S, and HT2 (A. McKnight and G. Singer, Brigham and Women's Hospital, Boston); FDC-P1 (S. Watowich, Whitehead Institute); 7TD1 and WEHI 279 (American Type Culture Collection); KD83 and transfected B16 lines producing various cytokines (G. Dranoff and J. Salter, Whitehead Institute), and MB49.1 (M. O'Donnell, Beth Israel Hospital, Boston). B16 producer lines were used as standards in the bioassays and controls in ELISAs. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used in each case (13).

For cytokine assays from splenocyte culture, supernatants from splenocytes that had been stimulated with medium alone, purified protein derivative (PPD), or Con A (each in triplicate for each experiment) were assayed by cytokine-specific ELISA. IL-2 was assayed at 24 hr after plating, and all other cytokines were assayed at 72 hr post-plating. Antibody pairs (all from PharMingen) for each cytokine were as follows: IL-2 (JES6-5H4 and JES6-1A12; sensitivity, 10 pg/ml); GM-CSF (MP1-2E9 and MP1-31G6; sensitivity, 40 pg/ml); IL-4 (BVD4-1D11 and BVD6-24G2; sensitivity, 50 pg/ml); IL-3 (MP2-8F8 and MP2-43D11; sensitivity, 10 pg/ml); IL-10 (JES5-2A5 and SXC-1; sensitivity, 100 pg/ml), and IFN-γ (Endogen ELISA mini-kit antibody pairs; sensitivity, 50 pg/ml). In some cases IL-10 and IFN-γ results were refined using more sensitive (10–20 pg/ml) premade kits supplied by Endogen as mentioned above. Horseradish peroxidase or alkaline phosphatase-coupled tertiary reagents were used to develop the ELISAs using standard procedures.

Injection of BCG into Mice. Female BALB/c, C57BL/6, and C3H/HeJ mice were purchased from The Jackson Laboratories, were 4–6 weeks old at arrival, and were injected within 10 days of arrival. All animal husbandry techniques were performed in accordance with the stipulations of the Massachusetts Institute of Technology Committee for Animal Care. Approximately 1 week prior to the injection date, an aliquot of each BCG strain was thawed and allowed to grow to an OD_{600} of 1–1.5. On the day of injection, the BCG samples were washed with phosphate-buffered saline (PBS) and resuspended in PBS to a roughly equal OD_{600} for all samples. Samples of the culture supernatants were stored at −70°C for later analysis of cytokine production. BCG strains were injected into the lateral tail vein or intraperitoneally in a total volume of 200 μl. After injection, a sample of the BCG was diluted and plated onto MH9 medium with kanamycin to determine the number of colony-forming units injected into each mouse. Results are shown from representative experiments that have been repeated several times.

***In Vitro* Antigen-Specific Responses.** Mice were sacrificed at weeks 6 and 16 postinfection in most experiments. For each time point, four to six mice were used. Spleens were removed aseptically and placed in RPMI medium containing 10% fetal calf serum, 50 μM 2-mercaptoethanol, glutamine at 30 μg/ml, and penicillin and streptomycin. The spleens were forced through a plastic mesh (70 μm; Falcon) to create a single-cell suspension. Splenocytes were purified on Ficoll (Pharmacia) according to standard procedures. After washing, the cells were placed in culture at a concentration of 4×10^6/ml in the same medium as above. For cytokine assays, a 1-ml volume was used, and for proliferation a 200-μl volume in 96-well plates was used. PPD was used at a final concentration of 20 μg/ml and was a generous gift of Connaught Laboratories. Con A was used at a final concentration of 5 μg/ml. For proliferation assays, cells were seeded in quadruplicate in 96-well plates with medium, PPD, or Con A in the concentrations described above. [^{3}H]Thymidine was added for the final 6 hr of a 72-hr incubation time. Cells were harvested on to glass-fiber filters, and incorporated radioactivity was measured.

RESULTS

Construction of BCG Recombinants That Secrete Cytokines. To construct BCG recombinants capable of secreting cytokines, selected murine cytokine cDNAs were modified by replacing the normal cytokine secretion signal-coding sequence with a mycobacterial secretion signal sequence (11). Murine IL-4, IL-6, GM-CSF, and IFN-γ cDNAs modified in this fashion were introduced into shuttle vectors in *E. coli*, and these vectors were subsequently introduced into BCG. Recombinant BCG clones were isolated and tested for their ability to secrete each cytokine into the medium by ELISA and by cytokine-specific bioassays. All of the cytokines could be detected by ELISA (Table 1), and all but IFN-γ were active in bioassays. Since BCG-IFN-γ has a significant effect in mice (see below) and IFN-γ is active in a homodimeric form (17), it seems likely that a small percentage of the IFN-γ secreted from BCG assembles into the active molecule, and this amount is below the detection limit of the IFN-γ bioassay. Thus, it appears that a broad spectrum of cytokines can be produced and secreted in an active form from BCG.

Cell-Mediated Immune Responses in Mice. The cytokine-secreting BCG strains were injected into BALB/c mice, as these mice are susceptible to BCG (BCG Montreal strain) infection and allow an early proliferation of bacteria in the liver and spleen (19, 20) but eventually control the infection. Mice were inoculated intravenously with 10^4–5×10^4 colony-forming units of bacteria and killed at 6 or 16 weeks postinfection; splenocytes were isolated and placed in culture either with no stimulus or with mycobacterial antigens (PPD). Antigen-specific proliferation and cytokine release were measured (Fig. 1). The 6-week time point was chosen because cell-mediated immunity is well established in all strains of mice (19, 20). The 16-week time point was chosen as the point at which any remaining BCG would be in homeostasis with the host. The antigen-specific response to PPD at 6 weeks postinfection was notable in that the BCG strain with the vector alone produced the highest response compared to the other strains. In several experiments we noted that the response at 6 weeks postinfection was often similar from strain to strain (see *Discussion*). The differences were apparent longer into the infection. Splenocytes harvested 16 weeks postinfection from mice infected with BCG secreting IL-2, GM-CSF, or IFN-γ showed a markedly enhanced response to PPD stimulation relative to control mice. Splenocytes from these mice showed enhanced proliferation, produced large amounts of IFN-γ, and secreted increased levels of other cytokines such as IL-2, IL-10, IL-3, and GM-CSF upon stimulation. No antigen-specific release of IL-4 was detected in any assays, although the cytokine was present in Con A-stimulated supernatants (data not shown). BCG strains secreting IL-6 or IL-4 were generally indistinguishable from BCG that do not produce cytokines in these assays.

The profound cellular responses to PPD in mice injected with BCG secreting IL-2, GM-CSF, or IFN-γ might be due to greater persistence of these bacteria relative to the other BCG

Table 1. Construction of recombinant BCG strains that secrete functional cytokines

BCG strain	Murine cytokine secreted	No. of disulfide bonds	Oligomerization*	Cytokine concentration,† pg/ml per OD_{600}	% activity‡
BCG-vector	—	—	—	—	—
BCG-IL-2	IL-2	1	Monomer	>5000	100
BCG-IL-6	IL-6	2?	Monomer?	2500	100
BCG-GM-CSF	GM-CSF	2	Monomer	1670	100
BCG-IL-4§	IL-4	3	Monomer	129	Active¶
BCG-IFN-γ‖	IFN-γ	0	Homodimer	7140	?**

*References for the structures of each cytokine are as follows: GM-CSF (14), IL-4 (15, 16), and IFN-γ (17). The structure of IL-6 has not been determined, and the number of disulfide bonds and oligomerization state is inferred (18). Where the structure is from the human protein, the mouse structure was taken by inference and inspection of the primary sequence for additional cysteine residues.

†Levels of cytokines produced by each strain as detected by cytokine-specific ELISA and normalized to an approximate cell density. The exact amount per cell cannot be determined due to the clumping properties of mycobacteria.

‡Bioassays using factor-dependent cell lines were used to determine if the cytokines secreted from BCG were biologically active. Supernatants from BCG-vector were used as negative controls in each case. The percentage of active cytokine secreted was calculated by titration against known amounts of recombinant cytokine.

§The BCG-IL-4 strain used in this study secretes an epitope-tagged version (10) of the protein. The epitope is created by the vector pRB4. Epitope-tagged versions produced more IL-4 than the untagged strains.

¶The percentage of biologically active IL-4 was difficult to determine because the cytokine detected was in the lower range of the CT.4S assay.

**Amounts of biologically active IFN-γ in the BCG supernatants were too low to detect in these assays.

recombinants over the course of the experiment. Alternatively, the results could be attributed to an enhanced cellular response to these cytokine-secreting BCG organisms. To distinguish between these possibilities, bacterial loads in the liver and spleens of mice were measured at 16 weeks postinfection. Less than 100–200 colony-forming units of BCG could be found per spleen or liver at the 16-week time point, regardless of the recombinant injected, and histological examination failed to reveal significant numbers of acid-fast bacteria in liver and spleen sections (data not shown). Hence, the mice had effectively cleared most of the BCG organisms, regardless of the BCG strain injected, and the increased antigen-specific responses of splenocytes are likely due to differences in the immunostimulatory properties of the IL-2-, GM-CSF-, or IFN-γ-secreting BCG rather than their persistence in mice.

BCG bacilli are disseminated throughout the body after intravenous injection, whereas they collect in the draining lymph nodes of the peritoneum and spleen after intraperitoneal injection. To determine if the stimulation observed with a standard intravenous injection of cytokine-secreting BCG occurs with an intraperitoneal injection, BALB/c mice were injected intraperitoneally with various BCG recombinants, spleens were removed at 16 weeks postinfection, and splenocyte responses were assayed as described above. Splenocytes from mice injected with the IL-2-, GM-CSF-, or IFN-γ-secreting BCG had an enhanced antigen-specific response, as measured by cytokine release and proliferation, compared to splenocytes from mice injected with the vector control. All cytokines measured were enhanced by administration of BCG-IL-2, BCG-GM-CSF, or BCG-IFN-γ, including IL-2, IFN-γ, and IL-10 (Fig. 2).

Response of Different Mouse Strains to BCG Secreting Cytokines. We investigated whether the enhanced response to cytokine-producing BCG occurs in two other inbred strains of mice, C3H/HeJ and C57BL/6. Splenocytes from C3H/HeJ mice that had been injected intraperitoneally with the BCG strains producing IL-2, GM-CSF, or IFN-γ were found to exhibit a strong response to *in vitro* PPD challenge when isolated 16 weeks after infection (Fig. 3). Enhanced antigen-specific responses were observed in the C3H/HeJ mice injected with cytokine-secreting BCG, and these involved increased production of IL-2, IFN-γ, and GM-CSF. C57BL/6 mice produced a much lower response to BCG and cytokine-secreting BCG than the other two mouse strains tested (data not shown), and the responses were too low to reach a conclusion regarding differences in the response to cytokine-secreting BCG. Nonetheless, the results with C3H/HeJ mice show that cytokine-secreting BCG can enhance immune responses in multiple strains of mice, indicating that the enhancement may be a general phenomenon and not restricted to a single inbred strain of mice.

DISCUSSION

Secretion of Active Cytokines from BCG. BCG strains have been constructed that produce a variety of cytokines fused to the BCG alpha antigen signal sequence and permit the secretion of these cytokines in a functional form. Thus far, BCG recombinants secreting functional IL-2 (10, 21), IL-4, IL-6, GM-CSF, and IFN-γ have been generated and characterized. These results demonstrate that *M. bovis* BCG are able to produce and secrete multiple cytokines, indicating that at least a portion of the secreted proteins are able to fold and assemble into the appropriate structures for activity. For the cytokine-secreting BCG studied to date, there is an inverse relationship between the number of intramolecular disulfide bonds predicted for the mature cytokine and the amount of cytokine detected by ELISA in the bacterial supernatants (Table 1). Cytokines whose active form have zero (IFN-γ), one (IL-2), or two (IL-6 and GM-CSF) disulfide bonds are more easily detected in BCG supernatants than cytokines having three disulfide bonds such as IL-4 and LIF (P.J.M., unpublished results).

Enhanced Cell-Mediated Immune Responses to Cytokine-Secreting BCG. Mice injected with BCG strains secreting GM-CSF, IL-2, and IFN-γ exhibited an enhanced antigen-specific T-cell response as measured by *in vitro* stimulation of splenocytes with PPD. All mice examined in this study had effectively cleared most of the BCG organisms by 16 weeks postinfection, regardless of the BCG strain injected, indicating that the increased antigen-specific responses of splenocytes are likely due to differences in the immunostimulatory properties of the cytokine-secreting BCG rather than their persistence in mice. The enhanced cell-mediated response to cytokine-

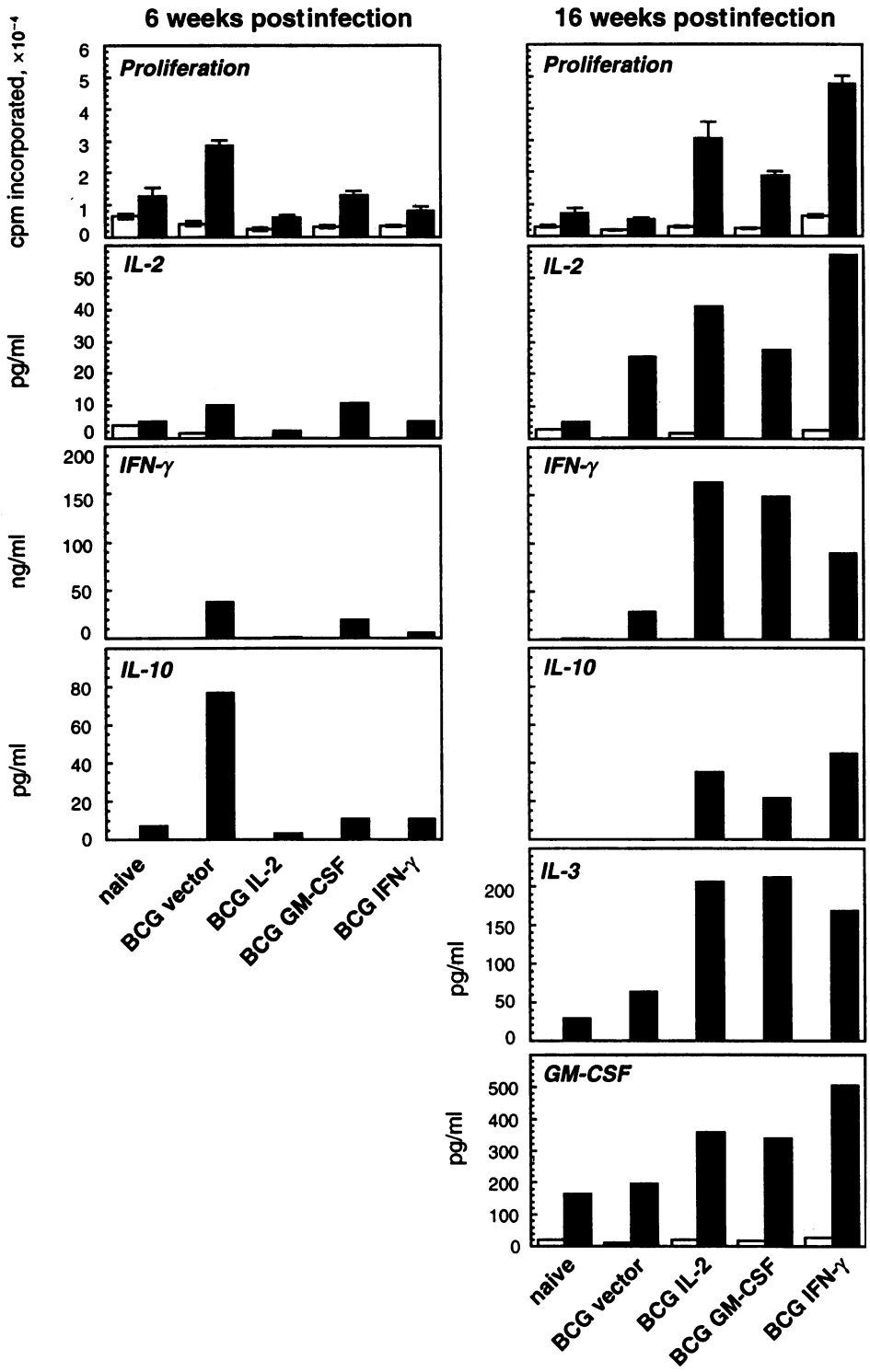

FIG. 1. Enhancement of anti-BCG responses after intravenous administration of recombinant BCG strains. *In vitro* proliferation and IL-2, IFN-γ, IL-10, IL-3, and GM-CSF secretion in response to medium alone (open bars) or PPD at 20 μg/ml (black bars) are shown from splenocyte pools from mice injected with each different strain of BCG (abscissa) and assayed at 6 weeks postinfection (*Left*) or 16 weeks postinfection (*Right*). Proliferation is shown as the average with the standard deviation of quadruplicate wells. Cytokine secretion in response to medium alone was undetectable in most cases. Cytokine assays are shown as the average of triplicate wells. For IL-2 secretion from stimulated splenocytes at the 16-week time point, only BCG-IFN-γ was assessed to be significantly different (paired *t* test; $P < 0.001$) than BCG-vector in two independent experiments including the one shown. The overall pattern of IL-2 secretion in response to PPD stimulation was similar in the two experiments.

secreting BCG strains was observed when mice were injected either by intravenous or by intraperitoneal routes, indicating that observed enhancement occurs independent of the route of inoculation. No signs of illness were observed with any BCG strains under any of the conditions used.

Enhanced cellular responses to cytokine-secreting BCG were observed in both BALB/c and C3H/HeJ mice, demonstrating that the effect is not restricted to a single inbred strain. BALB/c and C3H/HeJ mice differ in the rate at which they clear BCG after infection, where C3H/HeJ mice clear BCG more rapidly and are thus described as being relatively resistant to BCG infection (20, 22). Note that mortality is not observed in these mouse models of BCG infection. Parameters of resistance and susceptibility are measured by bacterial loads

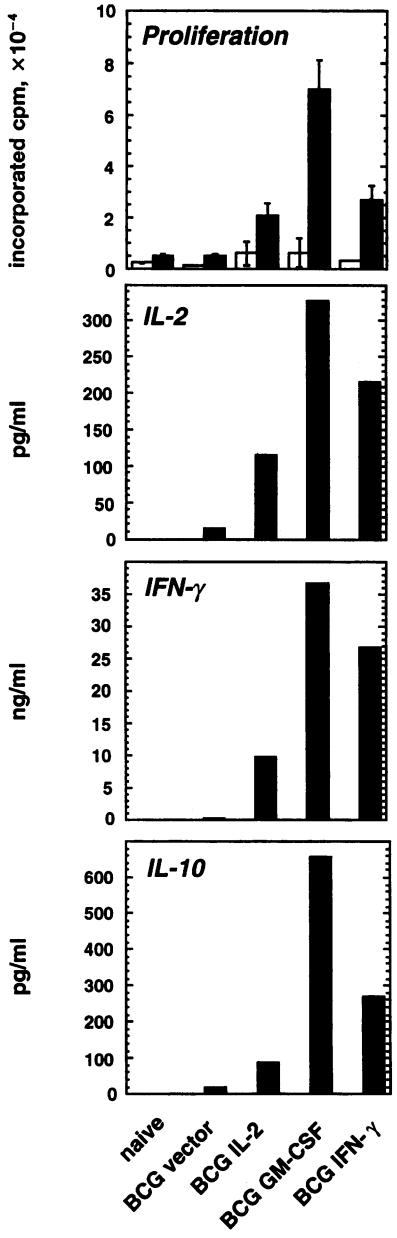

FIG. 2. Splenocyte cytokine secretion from mice injected intraperitoneally with different BCG strains. *In vitro* proliferation and IFN-γ, IL-2, and IL-10 secretion from splenocytes stimulated with medium alone (open bars) or PPD at 20 μg/ml (black bars) are shown. The BCG strains injected are shown along the abscissa. The figure depicts data from mice killed 16 weeks postinfection. Proliferation is shown as the average with the standard deviation of quadruplicate wells. Cytokine assays are shown as the average of triplicate wells.

at a given time point (19, 20). Despite these differences, both mice produced an enhanced antigen-specific T-cell response when injected with BCG strains secreting GM-CSF, IL-2, and IFN-γ. The timing of the cellular responses to each BCG strain appeared complex with an apparent lag in response to recombinant strains compared to the control (Fig. 1). The reasons for this are unknown at present but could be related to either the *in vivo* growth rate of the bacteria or the cytokine secreted influencing the immune response.

Interestingly, the cytokine secretion profiles of splenocytes from mice injected with cytokine-secreting BCG revealed enhanced levels of cytokines. Splenocytes from mice injected with cytokine-secreting BCG produced higher levels of IL-2 and IFN-γ, as well as IL-10, IL-3, and GM-CSF, relative to cells from mice exposed to the BCG-vector control. The enhanced

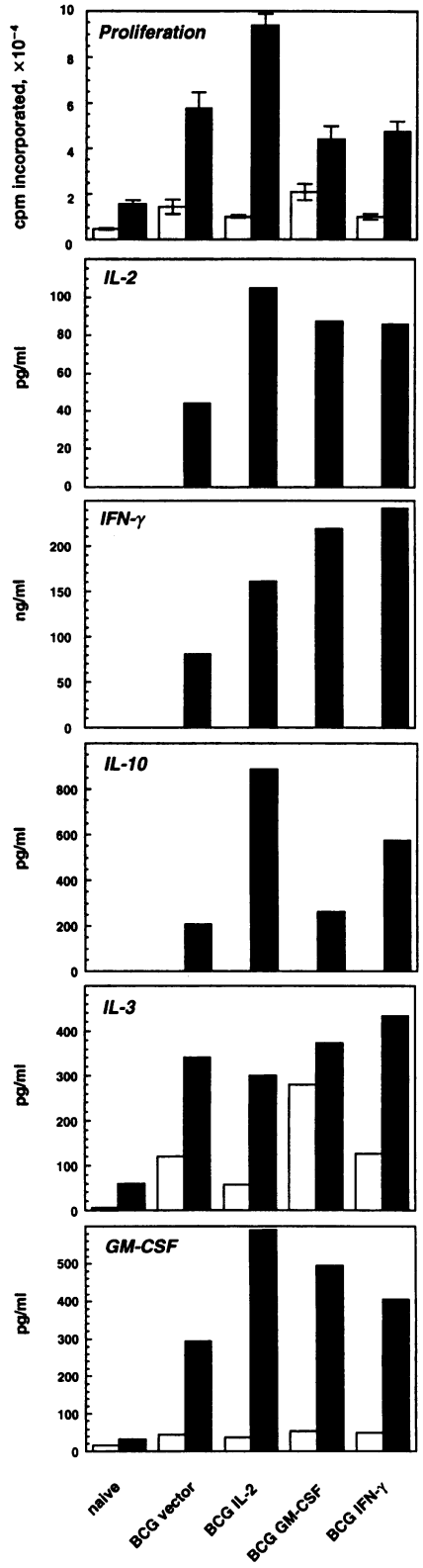

FIG. 3. Splenocyte cytokine secretion from C3H/HeJ mice injected intraperitoneally with different BCG strains. *In vitro* proliferation and IFN-γ, IL-2 IL-10, IL-3, and GM-CSF secretion from splenocytes stimulated with medium alone (open bars) or PPD at 20 μg/ml (black bars) are shown. The BCG strains injected are shown along the abscissa. The figure depicts data from mice killed 16 weeks postinfection. Proliferation is shown as the average with the standard deviation of quadruplicate wells. Cytokine assays are shown as the average of duplicate wells.

levels of these cytokines occurred in mice injected with BCG strains secreting GM-CSF, IL-2, or IFN-γ. It appears, there-

fore, that these cytokine-secreting BCG cause a quantitative change in the murine cellular response to mycobacterial antigens and do not modify the overall pattern of the cellular response as measured by the specific cytokine and proliferation assays. Limiting dilution analysis will be required to assess if the enhanced response is due to a corresponding increase in BCG-specific T-cell numbers or the increased activity of a subset of BCG-specific T lymphocytes. One explanation for the activities of the recombinant BCG is that they alter cytokine secretion from macrophages. Kaufmann and colleagues (23) have recently shown that BCG does not induce IL-12, an initiation cytokine for cell-mediated immunity, without prior stimulation with IFN-γ. Early exposure of macrophages to the cytokines delivered from BCG may enhance IL-12 secretion in the local environment of early infection sites.

Conclusions. BCG strains secreting cytokines, in particular GM-CSF, IL-2, and IFN-γ, can modify and potentiate the immune response to BCG antigens. These recombinants may be valuable for improved prophylactic and therapeutic vaccines and for further study of mycobacterial immune responses. The inclusion of a cytokine along with the use of small doses of BCG to trigger the induction of long-lasting cell-mediated immunity, an idea pioneered by Bretscher and colleagues (24, 25), may be an attractive candidate TB vaccine. BCG recombinants that express antigens of pathogens such as human immunodeficiency virus (8, 9), *Borrelia burgdorferi* (26), and *Leishmania* (27) engender long-lasting immune responses to the foreign antigens, and the coexpression of cytokines in these recombinants may further enhance desired immune responses. Cytokine-secreting BCG recombinants may also serve as improved reagents for bladder cancer immunotherapy, as there is a need for BCG strains that are effective at lower doses, and GM-CSF has already been shown to be a powerful cofactor in antitumor immunity experiments (28). Finally, BCG strains secreting cytokines may prove useful tools in dissecting the relationship between animals and mycobacteria, particularly in genetically altered mice, which themselves are modified to create systems that are more amenable to analysis of complex immune responses.

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