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Secretion of Human Interleukin 2 by Recombinant *Mycobacterium bovis* BCG

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The human interleukin 2 (huIL-2) gene was introduced into *Mycobacterium bovis* BCG by using the integrative vector pMV306. To express and secrete huIL-2 from BCG, two different plasmids, CI and CII, were made. In CI, the huIL-2-encoding region was under the control of the α-antigen promoter of BCG; in CII, the expression of huIL-2 was regulated by the heat shock protein 60 promoter. A signal peptide sequence isolated from the naturally secreted α-antigen of BCG was inserted between the promoter and huIL-2-encoding region to facilitate secretion. Both huIL-2 expression plasmids were integrated into the BCG genome when introduced into the BCG Pasteur strain by electroporation. Approximately 150 U of huIL-2 was secreted into the medium of a BCG-CII culture, while the BCG-CI cells secreted approximately one-sixth of that amount. When the IL-2-expressing BCG strain BCG-CII was injected intravenously into BALB/c mice, the number of BCG cells in the spleens of these mice was significantly less than the number in the control mice. The decreased number of IL-2-expressing BCG cells is likely due to the augmentation of the host immune response by the secreted huIL-2, although the exact mechanism is not known.

It has been estimated that 1 billion people in the world have been infected by *Mycobacterium tuberculosis* and approximately 3 million die of tuberculosis annually, especially in developing countries (31). Bacille Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, is the vaccine used for prevention of tuberculosis. Even though more than 2 million people are vaccinated with BCG annually, the efficacy is highly variable, from 0 to 80% (7). With the emergence of multiple-drug-resistant strains of mycobacteria, it is imperative to develop an improved vaccine for the prevention of mycobacterial infections. Despite the variable efficacy, BCG is an attractive vaccine vehicle due to a long history of safe use, an ability to act as an adjuvant, and the development of shuttle vectors to perform molecular manipulations (1). BCG has been used in expressing many foreign genes ranging from the *gag*, *env*, and *pol* genes of human immunodeficiency virus type 1 (2, 34) to a β-galactosidase gene from *Escherichia coli* (29). In addition, a B-cell epitope of the human immunodeficiency type 1 *gag* gene and an outer surface lipoprotein A (OspA) gene of *Borrelia burgdorferi* have been expressed and secreted from BCG as fusion proteins with the α-antigen of BCG (16, 28).

Although interleukin 2 (IL-2) was originally described as a T-cell growth factor (18), it was later found to play a pivotal role in the immune system and to have pleiotropic effects on different immune cells (25). IL-2 has been used in the treatment of various cancers (21) and has been suggested for the treatment of mycobacterial infections (12). It has been shown that administration of IL-2 in vivo can limit the replication of *Mycobacterium lepraemurium* (12) and *Mycobacterium avium* in mice (4). The toxicity associated with the use of high-dose IL-2 and its very short half-life in vivo limit its use (21). The expression and secretion of biologically functional IL-2 from recombinant BCG may overcome these problems. The secreted IL-2 may stimulate both humoral and cellular immune responses against mycobacterial or other recombinant proteins coexpressed in BCG to improve vaccine efficacy.

Recently, mouse and rat IL-2 proteins have been expressed and secreted from recombinant BCG strains by using the plasmid expression vector pMV261 (20). It was found that the IL-2-expressing BCG can enhance mouse gamma interferon production by splenocytes in vitro. Whether this result can be translated into in vivo efficacy remains to be seen.

In this paper, we report the expression and secretion of biologically active human IL-2 (huIL-2) from recombinant BCG along with in vivo data supporting the use of cytokine-secreting BCG to augment host immune responses against mycobacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotide primers. BCG Pasteur strains and *E. coli*-BCG shuttle plasmid vectors pMV261, pMV306, and pAB26 were kindly provided by Scott Koenig (MedImmune, Inc., Gaithersburg, Md.) (29). The 4-kb plasmid pMV306 is a derivative of pMV361 (29) in which the expression cassette was replaced by a multiple cloning site. Oligonucleotide primers were synthesized by using a DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). Primers DKU56 (TATgaattcGCCCGAATCGACATTTG; small letters indicate the added *Eco*RI site) and DKU57 (agtaggtgcCGCGCCCGCG GTTGCCGCTC; small letters indicate the sequence identical to the huIL-2 5′-encoding region) were used to synthesize a DNA fragment containing a promoter and signal peptide of α-antigen from BCG genomic DNA. DKU58 (ccgcgggcgcgGCACCTACTTCAAGTTCTAC; small letters indicate the sequence identical to the α-antigen signal peptide 3′ region) and DKU59 (AG GaagcttAATTATCAAGTTAGTGTTG; small letters indicate the added *Hin*dIII site) were the primers used to synthesize the huIL-2-encoding region. BCG cells were grown in Dubos media (Difco Laboratories, Detroit, Mich.) supplemented with 10% ADC (5% bovine serum albumin fraction V, 2% dextrose, and 0.85% NaCl).

Construction of expression plasmids. The plasmids used for expressing huIL-2 are shown in Fig. 1. Plasmid CI was constructed as follows. A DNA fragment containing the α-antigen promoter and signal peptide was synthesized by PCR with primers DKU56 and DKU57 from Pasteur strain BCG genomic DNA by adding an *Eco*RI site at its 5′ end and a 10-bp sequence identical to the huIL-2 5′-encoding region at its 3′ end. The huIL-2-encoding region was also synthesized by PCR with primers DKU57 and DKU59 from plasmid pCD-huIL-2 (32) by adding a *Hin*dIII site at its 3′ end and a 10-bp sequence identical to the α-antigen signal peptide 3′ region at its 5′ end. These two DNA fragments were spliced together by overlap extension with PCR using primers DKU56 and DKU59 as described previously (9), then digested with *Eco*RI and *Hin*dIII, and

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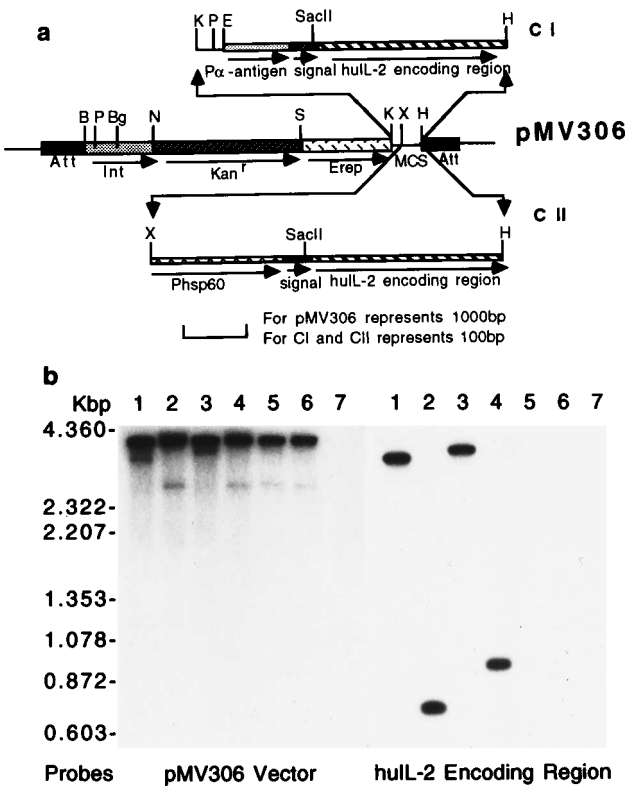

FIG. 1. (a) Construction of plasmids CI and CII used to express and secrete huIL-2 from the BCG Pasteur strain. Restriction enzyme sites shown: K, *Kpn*I; P, *Pst*I; E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; Bg, *Blg*II; N, *Not*I; S, *Sal*I; X, *Xba*I. MCS, multiple cloning site. (b) Southern hybridization of genomic DNA from BCG. BCG-CI (lanes 1 and 2), BCG-CII (lanes 3 and 4), BCG-pMV306 (lanes 5 and 6), and BCG (lane 7) were probed with pMV306 (left) and then with the huIL-2-encoding region (right), confirming the integration of construct pMV306-huIL-2 into the BCG genome. The genomic DNAs in lanes 1, 3, 5, and 7 were digested with *Kpn*I, while the DNAs in lanes 2, 4, and 6 were digested with *Kpn*I and *Hin*dIII.

cloned into pBluescriptSK+ (Stratagene Cloning Systems, La Jolla, Calif.). After the sequence was confirmed by DNA sequencing (22), the DNA fragment containing the α-antigen–huIL-2 sequence was cloned into the pMV306 *Kpn*I and *Hin*dIII sites located in the multiple-cloning-site region. Plasmid CII was derived from CI and plasmid pAB26 (28), which has the α-antigen gene linked to the heat shock protein (hsp60) promoter of BCG. Briefly, an *Xba*I-*Sac*II fragment having the hsp60 promoter and part of the α-antigen signal peptide sequence was obtained from pAB26, ligated to a *Sac*II-*Hin*dIII fragment having the whole IL-2-encoding sequence and part of the α-antigen signal peptide sequence from CI, and cloned into the *Xba*I-*Hin*dIII sites of pMV306.

Plasmids CI, CII, and pMV306 were transformed into the BCG Pasteur strain by electroporation as described previously (28). The transformed BCG cells were plated on Middlebrook 7H10 agar supplemented with 10% ADC and 15 μg of kanamycin per ml. After growth for 2 to 3 weeks at 37°C, individual colonies were picked and grown in Dubos media supplemented with 10% ADC.

Genomic DNA purification and Southern hybridization. Genomic DNA was isolated from BCG and recombinant BCG strains by a glass bead homogenization method as described previously (11). The genomic DNA was digested with the indicated enzymes and transferred to a nylon membrane. Southern hybridization was carried out with pMV306 as a probe; the product was stripped off by using boiling water and probed with the huIL-2-encoding region as described previously (27).

HuIL-2 bioassay. BCG-CII cells (2×10^8) were inoculated into 200 ml of Dubos medium supplemented with 10% ADC and grown in a 1,000-ml roller bottle at 37°C for 21 days. On day 3 and every 2 or 3 days subsequently, 10 ml of this culture was harvested. The bacterial cells were centrifuged at $12,000 \times g$, and the wet weight of the bacterial pellet was determined. The supernatant was filtered through a 0.45-μm-pore-size membrane. The huIL-2 activity from the supernatant was measured by using an IL-2-dependent HT-2 cell line with a colorimetric method as described previously (19). The IL-2 activity was converted into standard units as described elsewhere (8). To confirm that the activity in the supernatant of BCG-CII culture was due to huIL-2, 0.5 μg of goat anti-huIL-2 polyclonal antibody was used in a 200-μl HT-2 cell IL-2 assay.

Infection of mice. BALB/cCrAlt female mice (5 to 6 weeks old), obtained from the University of Alberta colony and given sterile food and acidified water, were inoculated intravenously through the tail vein with approximately 10^5 BCG, BCG-CI, BCG-CII, and BCG-pMV306 cells suspended in 0.1 ml of phosphate-buffered saline (PBS). At predetermined intervals, groups of mice (four to five mice per group) were sacrificed. The spleens from these mice were removed, weighed, and then homogenized in 5 ml of PBS solution. The bacteria in the spleens were diluted in PBS and then plated on Middlebrook 7H10 agar plates. After 2 to 3 weeks, the number of BCG colonies was counted. Differences among experimental groups were evaluated by Student's *t* test.

RESULTS

Construction of recombinant BCG strains expressing huIL-2. To express and secrete huIL-2 from BCG, two different plasmids (CI and CII) were made as described in Materials and Methods and shown in Fig. 1a. In CI, the huIL-2-encoding region was under the control of the α-antigen promoter of BCG, while expression of the huIL-2 gene was regulated by the hsp60 promoter in CII. A signal peptide sequence isolated from the naturally secreted α-antigen of BCG (17) was inserted between the promoter and the huIL-2-encoding region to facilitate secretion. In both plasmids, the huIL-2-encoding sequence was fused directly to the α-antigen signal peptide sequence by gene splicing with overlap extension (9), and the expression cassettes were cloned into the integrative vector pMV306 (29). pMV306 contains an attachment (*attP*) site and an integrase (*int*) gene derived from mycobacteriophage L5, which allows the integration of the vector into the BCG genome by site-specific recombination (14).

Plasmids CI, CII, and pMV306 were transformed into the BCG Pasteur strain by electroporation as described in Materials and Methods. In a typical experiment, approximately 500 to 1,000 colonies can be obtained from 1 μg of DNA transformed.

The integration of the huIL-2 gene and the vector pMV306 into the BCG genome generated three different BCG strains, BCG-CI, BCG-CII, and BCG-pMV306, in which plasmids CI, CII, and pMV306, respectively, were integrated into the BCG genome. Southern blot and hybridization results (Fig. 1b) obtained by using the huIL-2 gene and pMV306 as probes are consistent with the restriction map shown in Fig. 1a and indicate that all three plasmids integrated into the same region of the BCG genome as expected. A *Kpn*I site was located 0.3 kb to the left of the inserted DNA fragments, and another *Kpn*I site was located approximately 2 kb to the right.

Expression and secretion of huIL-2 from recombinant BCG. In the supernatant of a BCG-CII culture, a significant amount of biologically active huIL-2 was detected (Fig. 2a) and could be neutralized by goat anti-huIL-2 polyclonal antibody (Fig. 2b). An important distinction is whether the huIL-2 in the supernatant of the BCG-CII cultures is due to the release of huIL-2 from the autolysis of BCG cells or due to secretion. It has been shown that the autolysis of BCG cells generally occurs during the late log growth phase (33) and that the proteins released from the autolysis of BCG cells normally appear at this stage and then increase rapidly. To study the secretion of huIL-2 from recombinant BCG-CII, we monitored the growth of BCG-CII and the IL-2 activity in the supernatant of the liquid culture at various days. As shown in Fig. 2a, approximately 6 U of huIL-2 per ml was detected at 3 days prior to the log growth phase, and a rapid increase in the IL-2 level was then seen during the log growth phase. The huIL-2 activity in the supernatant corresponded closely with BCG-CII growth (Fig. 2a) and reached a plateau of approximately 150 U/ml. BCG-CI, with the α-antigen promoter instead of the hsp60

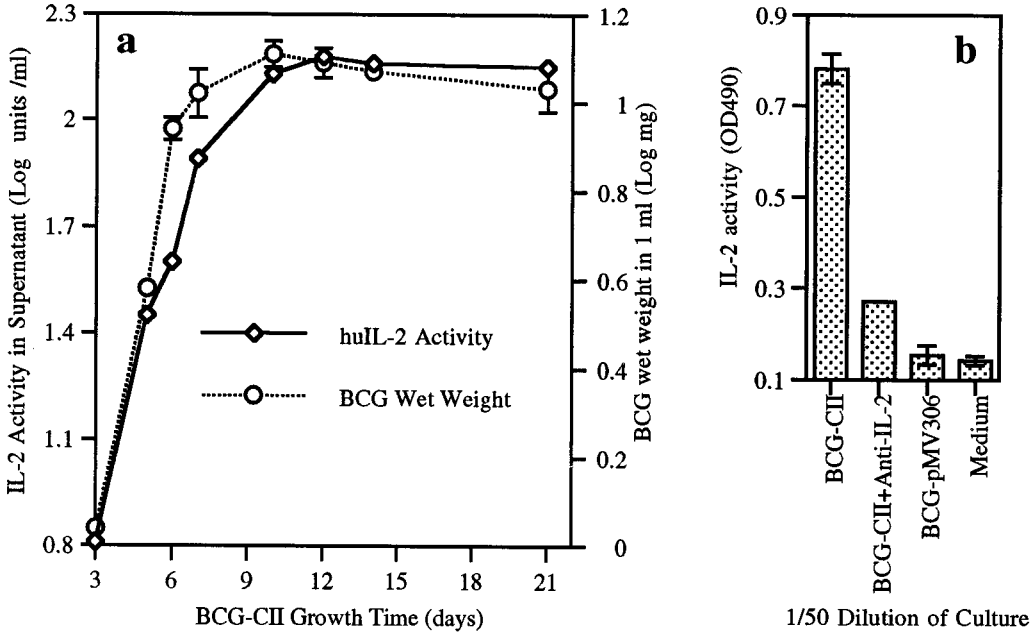

FIG. 2. Expression and secretion of huIL-2 from BCG-CII. (a) Growth of BCG-CII in Dubos medium and biological assay of IL-2 activity in BCG-CII supernatants. No huIL-2 activity was detected from the BCG or BCG-pMV306 control. (b) Neutralization of huIL-2 in BCG-CII supernatant harvested at day 21 with goat anti-huIL-2 polyclonal antibody. OD490, optical density at 490 nm.

promoter used in BCG-CII, expressed approximately one-sixth as much huIL-2 as did BCG-CII (data not shown).

Growth of huIL-2-expressing BCG in mice. To test whether the huIL-2-expressing BCG strains have any effects on the growth of BCG in vivo, we infected groups of BALB/cCrAlt mice intravenously with 1.1×10^5 CFU of various BCG strains and monitored the BCG growth in these mice. There was no difference in the seeding of the bacteria, as the number of BCG CFU in the spleens of mice injected with BCG-CII and the BCG-pMV306 control at 24 h after infection remained the same (Fig. 3c). At week 3, the viable bacterial counts in the spleens of mice infected with BCG-CII were significantly less than the counts in the spleens of mice infected with BCG ($P < 0.005$) or BCG-pMV306 ($P < 0.001$). Growth of the mycobac-

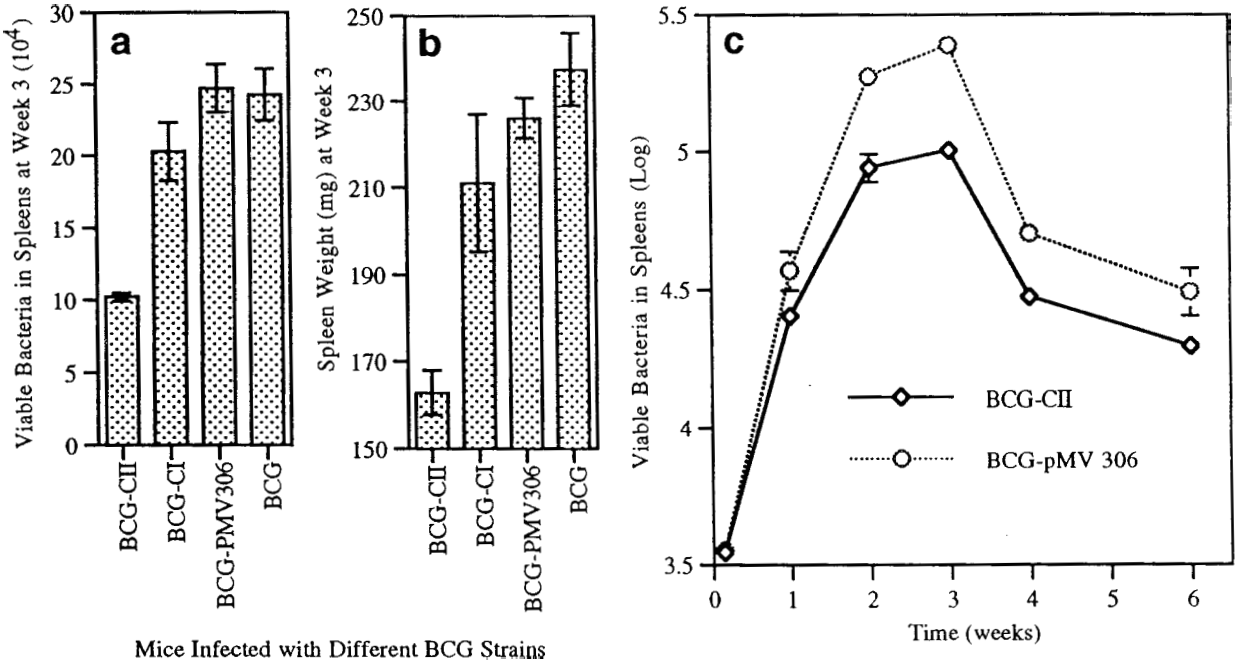

FIG. 3. Infection of BALB/c mice with Pasteur strain BCG, BCG-CI, BCG-CII, and BCG-pMV306. (a) Total viable bacteria counts in spleens at 3 weeks after infection. (b) Spleen weights of BALB/c mice infected with various BCG strains at week 3. (c) Time course of BCG-CII and BCG-pMV306 infection of BALB/c mice.

teria in the spleens correlated with splenic enlargement (Fig. 3b), consistent with a previous report (24). Starting from week 1 to the end of the experiment (week 6), the growth of bacteria was significantly less in the spleens of mice infected with BCG-CII compared with the control BCG-pMV306 (at week 1, $P < 0.05$) (Fig. 3c).

DISCUSSION

Initially, an autonomously replicating plasmid vector, pMV261 (29), was used unsuccessfully in an attempt to express huIL-2. When plasmid pMV261, having either the huIL-2 gene or the huIL-2 gene with the α-antigen signal peptide under the control of hsp60 promoter, was electroporated into BCG, no colonies grew on the Middlebrook 7H10-kanamycin selection plates, while 1 μg of pMV261 control DNA generally gave rise to 500 to 1,000 colonies (data not shown). Thus, the huIL-2 gene could not be expressed in the BCG Pasteur strain with the shuttle plasmid vector pMV261. On the other hand, the huIL-2 gene, with or without the α-antigen signal sequence, can be maintained in the genome of BCG. This provides an example of the maintenance of a foreign gene, which was not easily established with plasmid vectors, by using an integrative vector in BCG just as described for other bacterial systems (23). A likely explanation for this phenomenon is overexpression lethality (5).

Mouse IL-2 and rat IL-2 have recently been expressed with the plasmid vector pMV261 (20). It was also found that the BCG transformation efficiencies for pMV261 having the mouse and rat IL-2 genes without signal sequences were 10- to 100-fold less than those of the IL-2 constructs having the α-antigen signal sequence. The difference in the ability to express the murine and human IL-2 genes with pMV261 in BCG may be due to the IL-2 sequence diversity between these species (35). The predictors of success for expressing proteins in BCG with these expression vectors are currently unknown, although expression seems to be somewhat gene and protein specific (13a). With the use of the integrative vector pMV306, the huIL-2 gene in both plasmids CI and CII was integrated into the BCG genome. The Southern hybridization results show that CI, CII, and the pMV306 vector control were integrated into the same chromosomal region of BCG. These results are consistent with previous studies (14), which showed that the phage L5-based vectors integrate site specifically into a tRNA region of the mycobacterial genome. This integration does not appear to disrupt any essential function of any gene (14).

In the supernatant of a BCG-CII culture, approximately 150 U of huIL-2 per ml can be detected by using the HT-2 bioassay. More than 95% of huIL-2 activity was present in the medium, while less than 5% of huIL-2 activity was detected from the BCG-CII cell sonic extracts (data not shown), and it has been shown that the extracts of BCG did not contain any IL-2 inhibitor (20). The huIL-2 activity can be detected prior to log-phase growth and corresponded closely with BCG-CII growth (Fig. 2a). Consistent with previous studies on the secretion of proteins from BCG and *M. tuberculosis* (33), the huIL-2 detected from the BCG-expressing strain culture is due to the secretion of huIL-2 from BCG cells and not due to the autolysis of BCG cells.

BCG-CI, in which the huIL-2 gene was under the control of BCG α-antigen promoter instead of BCG hsp60 promoter used in BCG-CII, expressed approximately one-sixth the amount of huIL-2 expressed by BCG-CII. This result indicates that the BCG hsp60 promoter is a better promoter than the BCG α-antigen promoter in driving expression and secretion of huIL-2. Consistent with this observation, we have found that the hsp60 promoter is also a stronger promoter than the BCG α-antigen promoter in directing the expression of other cytokines (13b).

The heat shock protein promoters are the most commonly used promoters in expressing foreign genes in mycobacteria (5). The expression of hsp60 protein is constitutive under all growth conditions and is highly induced with moderate heat shock (37°C) (36). With the use of the hsp60 promoter, foreign genes can be expressed to 10% or more of the total mycobacterial protein in BCG (29). Even though the α-antigen promoter directs the expression and secretion of large amounts of α-antigen proteins during the early phases of growth of mycobacteria (36), we found that the α-antigen promoter serves as a poor promoter in directing the expression of huIL-2 in the BCG Pasteur strain. The reasons for this discrepancy are unknown. Since the promoter of the α-antigen was deduced only from sequence comparison (17), the exact DNA sequences involved in the regulation of α-antigen expression are unknown. As only 90 bp of the 5′ regulatory sequences of the α-antigen are included in our promoter, it is possible that some regulatory sequences required for high expression of the α-antigen are missing from the promoter that we used.

It has been shown that IL-2 plays an important role in mycobacterial infections and that the administration of IL-2 in mice can make mice more resistant to mycobacterial infections (4, 12). Therefore, we studied the growth of the huIL-2-expressing BCG in the spleens of mice to test whether the huIL-2-expressing BCG can limit the growth of BCG in vivo. We found that starting from week 1 to the end of experiments, the bacterial numbers in the spleens of mice infected with BCG-CII were significantly less than those in the spleens of mice infected with various control BCG strains (Fig. 3). This difference is not likely due to different growth rates of the various BCG strains, as there was no difference in the growth rates of these strains in vitro (data not shown). If the actual insertion of pMV306-huIL-2 affected the in vivo growth rate, the same effect would be expected with control BCG-pMV306, in which the vector pMV306 was inserted into the same region as BCG-CII. There was no significant difference in the bacterial counts of mice infected with BCG and BCG-pMV306. Moreover, when the same number of BCG-CI, which expresses approximately one-sixth the amount of huIL-2 as BCG-CII, was injected into the mice, the number of bacterial CFU recovered from the spleens of these infected mice was significantly less than the number of CFU recovered from mice infected with BCG ($P < 0.05$; Fig. 3a) or BCG-pMV306 ($P = 0.01$; Fig. 3a). It was, however, significantly greater than the number recovered with BCG-CII ($P < 0.01$ Fig. 3a). The lower number of CFU of the huIL-2-expressing BCG strains recovered from the spleens of infected mice is likely due to the enhancement of the host immune response by the secreted huIL-2. The immune mechanisms involved in this process, particularly with respect to T cells and macrophages, are under investigation.

M. tuberculosis is an intracellular pathogen that lives and multiplies in nonactivated macrophages. Before *M. tuberculosis* can be destroyed by macrophages, these cells must be activated by T cells and/or lymphokines. IL-2 secreted from the IL-2-expressing BCG may activate macrophages directly (15, 30) or indirectly, by inducing the production of gamma interferon (13) and other cytokines (3). It has been shown that recombinant BCG secreting mouse or rat IL-2 can increase the gamma interferon production of mouse spleen cells in vitro (20). The IL-2-expressing BCG may be a more effective vaccine for tuberculosis or may simply serve to help us to better understand the immune response to BCG.

BCG instillation is the treatment of choice for recurrence of

superficial bladder cancer (26). The combination of IL-2 and BCG in the therapy of superficial bladder cancer is more effective than either IL-2 or BCG alone in both a mouse model (10) and a clinical trial (6). Therefore the IL-2-expressing BCG-CII may also have a role in the treatment of bladder cancer.

Recombinant cytokine-expressing BCG provides a model system for studying immunity to mycobacterial infections and, in particular, the roles of various cytokines. This system will provide information that will be invaluable in the development of immune modulators for the prevention and treatment of tuberculosis.

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