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ARTICLES

New use of BCG for recombinant vaccines

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BCG, a live attenuated tubercle bacillus, is the most widely used vaccine in the world and is also a useful vaccine vehicle for delivering protective antigens of multiple pathogens. Extrachromosomal and integrative expression vectors carrying the regulatory sequences for major BCG heat-shock proteins have been developed to allow expression of foreign antigens in BCG. These recombinant BCG strains can elicit long-lasting humoral and cellular immune responses to foreign antigens in mice.

VACCINES are the most cost-effective medical intervention known to prevent disease^{1,2}. Although two million children's lives are saved each year by immunization^{3,4}, it will be very difficult to protect more children in the poorest countries with existing technology. Significant problems inherent in existing vaccines include the need for repeated immunizations, reduced effectiveness due to maternal antibodies in children in the vulnerable first months of life, the need for injection, heat instability and cost. One strategy which might overcome some of these limitations is the development of recombinant vaccine vehicles which allow simultaneous expression of multiple protective antigens of different pathogens.

BCG (bacille Calmette-Guérin), a live attenuated bovine tubercle bacillus used to immunize against tuberculosis⁵, offers some unique advantages for developing such a multivaccine vehicle: (1) BCG is the most widely used vaccine in the world, having been given to over 2,500 million people since 1948, with a low incidence of serious complications (case fatality rate of 0.19/10⁶)⁶; (2) it can be given at or any time after birth, and is

unaffected by maternal antibodies; (3) BCG is given as a single inoculum and sensitizes to tuberculo-proteins for 5-50 years; (4) it is a potent adjuvant in experimental animals and man^{7,8}; (5) BCG can be administered as an oral vaccine; (6) it is the most heat stable of live vaccines; and (7) it is inexpensive to produce.

Multicopy and integrating shuttle vectors

First generation *Escherichia coli*-mycobacterial shuttle vectors, including temperate phages and plasmids, were used to demonstrate the feasibility of transforming and propagating recombinant DNA in *Mycobacterium smegmatis* and BCG^{9,10}. We found these first-generation vectors, for example plasmid pYUB12, to be unsuitable for the introduction and expression of foreign genes in mycobacteria because they were large, lacked useful unique restriction sites and, in the absence of antibiotic selection, were unstable in BCG. However, we have now identified a 1.8-kilobase (kb) segment (*oriM*) from plasmid pAL5000¹¹ which supports plasmid replication in *M. smegmatis* and BCG. Modification of *oriM* by polymerase chain reaction (PCR) mutagenesis was done to eliminate undesirable restriction sites and an *oriM* cassette was then used as the basis for developing second-generation extrachromosomal vectors using three additional PCR cassettes: (1) the *E. coli* plasmid replicon derived from pUC19 (*oriE*); (2) the Tn903-derived *aph* gene conferring kanamycin resistance (*kan*^r) as a selectable marker; (3) an expression cassette containing a mycobacterial promoter, a multiple cloning site and a transcriptional terminator. The resulting *E. coli*-mycobacteria shuttle vector (Fig. 1a) were shown to transform BCG at high efficiencies (10⁵-10⁶ c.f.u. per µg DNA) and maintain about five plasmid copies per genome equivalent (Fig. 1b).

An advantage of BCG is its innocuous persistence *in vivo* that could provide continuing immunization to recombinant antigen. However, the vectors carrying antigen genes need to be stably

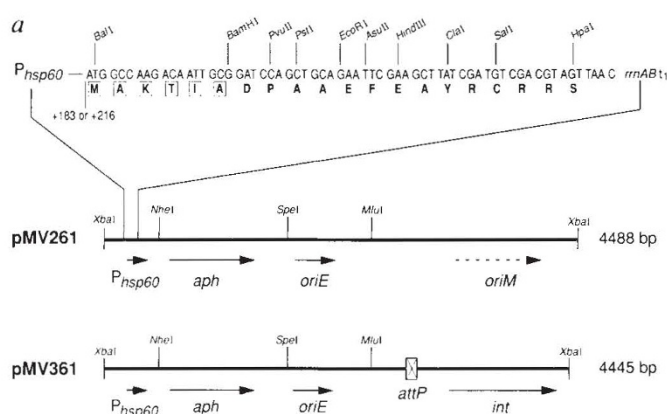
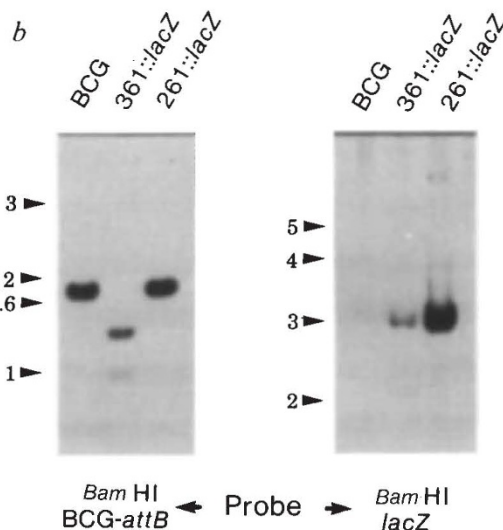


FIG. 1 *a*, BCG extrachromosomal (pMV261) and integrative (pMV361) expression vectors. Plasmids pMV261 and pMV361 share common elements including an expression cassette, the Tn903-derived *aph* gene conferring kanamycin-resistance as a selectable marker, and an *E. coli* origin of replication (*oriE*) derived from pUC19. They differ by the inclusion of either a mycobacterial plasmid origin of replication (*oriM*) or the *attP* and *int* genes of mycobacteriophage L5. The expression cassette contains 404 bp of the 5' regulatory region of the BCG *hsp60* gene³¹, including the first six amino acids (in boxes, single-letter amino-acid notation), 10 unique cloning sites and the *rrnABT1* transcriptional terminator³² aligned in the same transcriptional orientation as the other elements. *b*, Comparison of extrachromosomal (pMV261) and integration-proficient (pMV361) vectors. The *E. coli lacZ* gene was cloned into the unique *Bam*HI sites of pMV261 and pMV361 to form fusion proteins with *hsp60* and transformed into the Pasteur strain of BCG



as described previously¹⁰. Chromosomal DNAs were isolated from BCG and BCG transformants, digested with *Bam*HI, and Southern blot analyses were performed probing with either the BCG *attB* (ref. 13) gene or *lacZ*. Probing with *attB* (left panel) demonstrates that the pMV361::lacZ is integrated into the BCG chromosome as seen by the resolution of the *attB* sequence into an *attL* and *attR* fragments. Probing with the *lacZ* gene (right panel) demonstrates that pMV261::lacZ has a copy number of five as quantitated by an AMbis Direct Radioactivity Detector relative to the pMV361::lacZ site-specific integrant.

maintained *in vivo*. Selections for maintenance of plasmids *in vivo* which are useful in other bacterial systems¹² have not yet been adapted for the mycobacteria and as an alternative approach we have exploited the site-specific integration system of a temperate mycobacteriophage¹³. The integration-proficient vector, pMV361, was constructed by replacing the *oriM* region of pMV261 with a DNA segment carrying the attachment site (*attP*) and the integrase (*int*) gene (Fig. 1*a*) from the mycobacteriophage L5¹³. BCG is transformed with pMV361 through a site-specific integration into the chromosomal *attB* site with a similar efficiency to that of extrachromosomal vectors. Because the phage *xis* gene is not present the integrated vectors are stably maintained even without antibiotic selection¹³. Compatibility with *oriM*-containing plasmids also adds flexibility to these vector systems.

Foreign antigen expression in BCG

Although little is known about mycobacterial gene expression, particularly in the intracellular environment of the host, the abundant and highly conserved *hsp60* and *hsp70* cognate stress proteins are major antigens of mycobacteria, rickettsia, coxiella and chlamydia¹⁴⁻¹⁸. The Hsp60 and Hsp70 proteins are essential under all growth conditions, but are expressed at higher amounts in response to stress¹⁹. Thus the regulatory sequences of the BCG *hsp60* and *hsp70* genes were chosen to drive the expression of foreign antigen genes in BCG. Primer extension analysis identified two shock-responsive transcription start sites for the *hsp60* (Fig. 2*a*) and *hsp70* (data not shown) genes which had not been characterized previously. Endogenous BCG Hsp60 and Hsp70 protein levels also increased in response to stress with heat and acid but not peroxide (Fig. 2*b*).

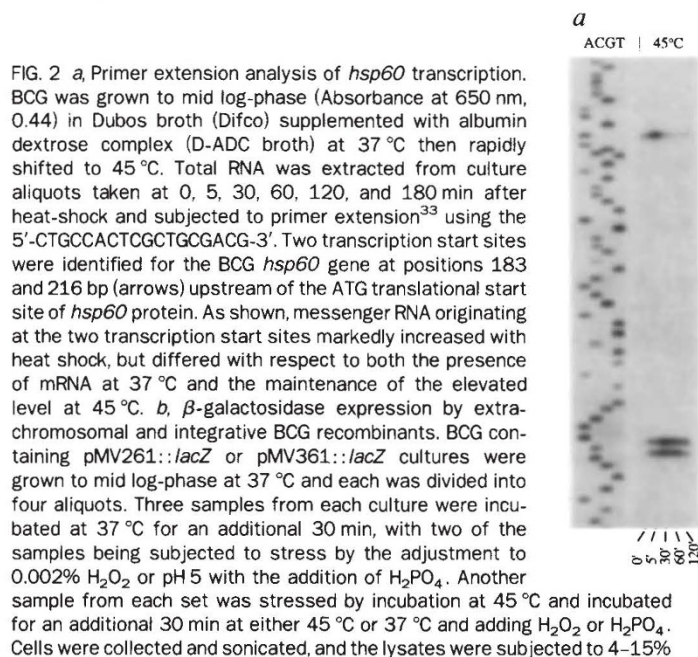
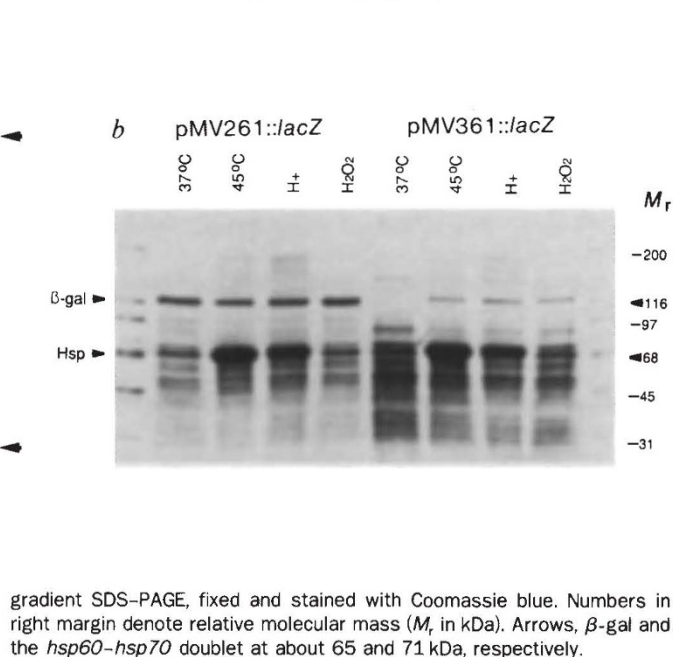


FIG. 2 *a*, Primer extension analysis of *hsp60* transcription. BCG was grown to mid log-phase (Absorbance at 650 nm, 0.44) in Dubos broth (Difco) supplemented with albumin dextrose complex (D-ADC broth) at 37 °C then rapidly shifted to 45 °C. Total RNA was extracted from culture aliquots taken at 0, 5, 30, 60, 120, and 180 min after heat-shock and subjected to primer extension³³ using the 5'-CTGCCACTCGCTGCGACG-3'. Two transcription start sites were identified for the BCG *hsp60* gene at positions 183 and 216 bp (arrows) upstream of the ATG translational start site of *hsp60* protein. As shown, messenger RNA originating at the two transcription start sites markedly increased with heat shock, but differed with respect to both the presence of mRNA at 37 °C and the maintenance of the elevated level at 45 °C. *b*, β -galactosidase expression by extrachromosomal and integrative BCG recombinants. BCG containing pMV261::lacZ or pMV361::lacZ cultures were grown to mid log-phase at 37 °C and each was divided into four aliquots. Three samples from each culture were incubated at 37 °C for an additional 30 min, with two of the samples being subjected to stress by the adjustment to 0.002% H_2O_2 or pH 5 with the addition of H_2PO_4 . Another sample from each set was stressed by incubation at 45 °C and incubated for an additional 30 min at either 45 °C or 37 °C and adding H_2O_2 or H_2PO_4 . Cells were collected and sonicated, and the lysates were subjected to 4-15%



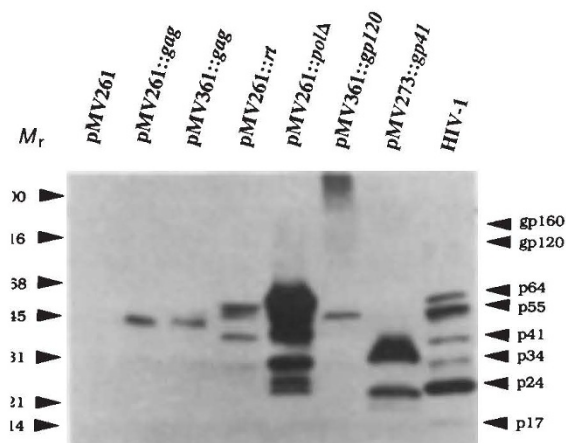


FIG. 3 Expression of HIV-1 antigens (IIIB isolate) in recombinant BCG. Genes encoding HIV-1 *gag*, *rt* (reverse transcriptase), *polΔ* (polymerase minus protease), *gp120*, and *gp41* proteins were amplified by PCR, fused to the sixth codon of the *hsp60* gene in pMV261 or pMV361, or the HSP70 gene³⁷ in pMV273 and transformed into BCG. Lysates of recombinant BCG were analysed for expression by western blot analysis of 4–20% gradient SDS-PAGE using human sera from a patient infected with HIV-1 and ¹²⁵I-labelled *Staphylococcus aureus* protein A. Arrows in left margin denote *M_r* standards. Arrows in right margin indicate apparent *M_r* (in kDa) of HIV-1 proteins.

The *E. coli lacZ* genes was placed under control of the BCG *hsp60* regulatory sequences to evaluate its potential for expressing foreign genes. Striking constitutive expression of the *hsp60* driven *lacZ* gene, in excess of 10% of total BCG protein, was observed from the extrachromosomal vector (Fig. 2b, pMV261::*lacZ*). Expression of *hsp60*::*lacZ* from the integrative vector was lower than that from the extrachromosomal vector; however, expression from the integrative vector, in contrast to the expression from the extrachromosomal vector, showed a coincident increase in Hsp60-β-gal in response to stress with heat, acid, and peroxide (Fig. 2b).

Antigen genes from a variety of pathogens including viral, parasitic and bacterial pathogens were cloned and expressed in recombinant BCG (rBCG). Several of the antigens of human immunodeficiency virus type 1 (HIV-1), which are relatively difficult to express at high amounts in other bacterial systems, were expressed in rBCG. Although expression of the HIV-1 *pol* coding region (minus protease) was substantial, expression

quantities for most of the *hsp60*-driven HIV-1 antigen genes did not rival those of *hsp60*-β-gal (Fig. 3). Extrachromosomal expression sometimes appeared to be lethal and it was only possible to express foreign genes in the single-copy integrative vectors (for example HIV-1 gp120). In other cases extrachromosomal and integrative expression were comparable (for example HIV-1 *gag*, Fig. 3). These studies establish the value of the BCG *hsp* regulatory sequences in driving expression of foreign genes in mycobacteria and suggest that homologous *hsp* sequences may be generally useful for enhancing expression of foreign genes in other recombinant vaccines.

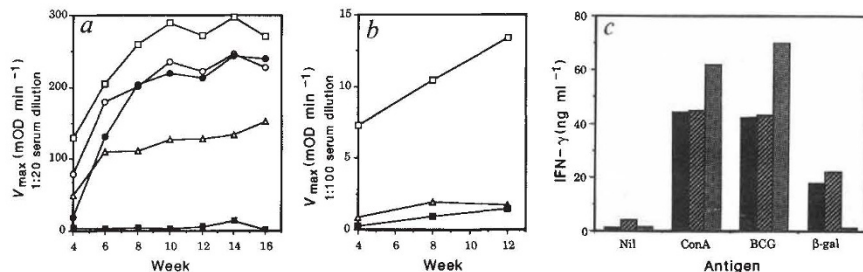
Immune responses to model antigens

To investigate the immunological capabilities and possible limitations of rBCG vaccines, we examined the humoral and cellular immune responses to several antigens expressed in BCG. Mice (BALB/c) were immunized with a rBCG expressing β-gal on a plasmid as a fusion protein with the first six amino acids of BCG *hsp60* antigen. Antibodies to β-gal were found in mice inoculated with the rBCG by all routes (Fig. 4a). Most striking was that very low numbers of live rBCG (as few as 200 c.f.u. given intravenously, and 10⁴ c.f.u. intradermally) were sufficient for high levels of antibody to β-gal (Fig. 4a). After a delay, antibody levels were sustained for many weeks. Antibodies to β-gal were not only higher (ELISA titre of 1:30,000, Fig. 4a) than responses to BCG sonicate (titre 1:5,000), but persisted much longer than those to BCG, which peaked only 2–4 weeks after immunization and waned thereafter. Thus greater antibody responses may be achieved to recombinant proteins expressed by rBCG than to antigens of BCG itself.

The growth of rBCG and the stability of the extrachromosomal plasmid without *in vivo* drug selection were established by the recovery of rBCG from mouse spleens 2–4 weeks after immunization. All BCG colonies were kan^r and expressed β-gal, and the number of bacilli recovered was >30 times that estimated to be localized in the spleen from the original inoculum. Viable rBCG recovered from spleen diminished between 2 and 4 weeks which is similar to non-recombinant BCG, but all rBCG recovered remained kan^r and expressed β-gal. These results, coupled with the delayed but sustained anti-β-gal antibody responses, establish that *hsp60*-driven expression of *lacZ* was effective for the expression of this model antigen *in vivo*, and suggest that actively growing rBCG can provide continuous boosting to the foreign antigen from a single immunization.

To assess the humoral immune response to an antigen expressed at substantially lower levels than β-gal in BCG, mice

FIG. 4 a, Antibody production to β-galactosidase in mice immunized with rBCG::*lacZ*. The rBCG was grown to mid log-phase in Dubos-ADC broth, then concentrated 20-fold by centrifugation. The concentrated bacteria were resuspended in PBS + 0.05% Tween 80 and cup sonicated briefly to disperse clumped bacteria. Six-week-old BALB/c mice were inoculated with a single dose of 2 × 10², 2 × 10⁴ or 2 × 10⁶ c.f.u. (determined after inoculation in each experiment) by either intravenous (i.v.), intradermal (i.d.), or intraperitoneal (i.p.) routes. Sera were obtained biweekly, pooled and analysed by



a β-gal-specific enzyme-linked immunosorbent assay (ELISA) with horse radish peroxidase-conjugated goat anti-mouse immunoglobulin and ABTS colour developing reagent (Kirkegaard and Perry). Colour reactions were read kinetically over 3 min on a *V_{max}* (maximum reaction velocity) plate reader (Molecular Devices) and results are presented as *V_{max}* rates (mOD min⁻¹). *V_{max}* rates of 200 mOD min⁻¹ and 300 mOD min⁻¹ were equivalent to standard ELISA titre determinations of about 1:10,000 and 1:30,000, respectively. □, 10⁶ i.v.; ○, 10⁶ i.p.; △, 10⁴ i.d.; ●, 10² i.v.; ■, naive. b, Antibody production to tetanus toxin fragment C in mice immunized with rBCG::*tox*C. DNA encoding fragment C of *Clostridium tetani* toxin (*tox*C) was cloned and fused in frame with the sixth codon of BCG *hsp60* into pMV261. NIH swiss mice were inoculated with predetermined c.f.u. by the

indicated routes. Parallel inoculations were made with the same preparations that had been heat killed for 30 min at 70 °C. Sera were obtained monthly, pooled and analysed at 1:100 dilution by ELISA as above using plates coated with 10 μg ml⁻¹ tetanus toxoid (Connaught Labs). □, Live 10⁶ i.d.; △, dead 10⁶ i.d.; ■, naive. c, INF-γ production by lymphocytes from mice immunized with rBCG::*lacZ* and rBCG::*gp120*. Splenocytes (ACK-treated 5 × 10⁶ ml⁻¹, where ACK is ammonium chloride/potassium carbonate) were cultured in 0.5 ml 50% RPMI 1640 medium, 50% EHAA medium (Gibco), 10% FCS supplemented with: media (Nil); concanavalin A, 5 μg ml⁻¹; WHO BCG Vaccine, 100 μl ml⁻¹ culture; or β-galactosidase, 10 μg ml⁻¹. IFN-γ levels in supernates from 72 h cultures were determined by ELISA³⁴. Solid bars, 10² rBCG *lac* (i.v.); hatched bars, 10⁶ rBCG::*lacZ* (i.p.); dotted bars, rBCG::gp 120 (i.p.).

were immunized with rBCG expressing the fragment C (ToxC) of tetanus toxin²⁰. This fragment (relative molecular mass, 50,000) lacks toxic activity, but binds to the cellular glycolipid receptor. ToxC expression was 1/250th that of β -gal on a weight basis (1/100 on a molar basis). Immunization with a single dose of rBCG::toxC led to antibody levels that increased for at least 12 weeks (Fig. 4b). The ability of rBCG to induce antibodies is therefore not unique to β -gal or to antigens expressed only at high levels. Heat-killed BCG::toxC was not effective at immunization, supporting the view that the persisting immune responses with rBCG are due to growth and persistence of the vaccine. That IgG responses were obtained implies that T cells also responded to the rBCG. This was confirmed by assessment of cellular immune responses. Lymphokines, specifically interferon- γ (IFN- γ), were produced *in vitro* by spleen cells stimulated with β -gal obtained only from animals inoculated with rBCG::lacZ, and not with rBCG::gp120, (Fig. 4b).

Cytotoxic T lymphocytes (CTL) are critical for resistance to many viral and bacterial infections²¹. There was much cytotoxic activity in splenocytes from mice immunized with 2×10^6 , or as few as 200 c.f.u. rBCG::lacZ given 19 weeks earlier (Fig. 5a-c). To learn whether the induction of CTL precursors is likely to be a general property of rBCG vaccines or whether it is peculiar only to β -gal or to antigens expressed at very high levels, lymphocytes from mice immunized with rBCG expressing HIV-1 gp120 antigen from pMV361 were examined for cytotoxic activity against P815 target cells pulsed with the P18 peptide. This peptide is the major CTL epitope of HIV gp120 in BALB/c mice and killing has been shown to be restricted to the major histocompatibility complex (MHC) class I D^d allele²². There was notable specific lysis (Fig. 5d), confirming the induction of CTL to the gp120 peptide. The failure to detect killing of the target cells in the absence of the peptide and the fact that our P815 cells did not express MHC class II antigens, suggest that lysis is mediated by MHC class I-restricted CTL.

Discussion

The ultimate goal in the development of vaccines is to induce sustained immunity against multiple pathogens with the minimum number of inoculations. Whether any single vehicle or delivery system can effectively evoke the required immune responses to a wide variety of antigens in a single dose remains to be seen. Nevertheless, it has been possible to express, in addition to the three antigens described here, antigens from 17 viral, bacterial and parasitic pathogens in rBCG. All the immunological data presented here derive from single-dose immunizations. Although notable amounts of antibodies were produced and maintained to both β -gal and to ToxC, the lack of significant titres (>1:100 dilution) of antibodies found to HIV gp120 suggests that the level of expression may be important. In the case of both β -gal and ToxC, antibodies were induced by the intradermal route used for human immunization. It is not evident how an intracellular organism whose recombinant protein would presumably be subjected to proteolysis within the lysosomal compartment of macrophages, can effectively elicit antibody responses, which generally recognize epitopes on intact antigens. Because under standard conditions for culture of BCG vaccine substrains only 25–40% of acid-fast bacilli are able to form colonies²³, the rBCG vaccines must contain nonviable organisms capable of releasing relatively intact recombinant antigens recognized by B cells. Nevertheless, killed rBCG expressing ToxC failed to immunize, indicating that dead bacilli are not effective immunogens. This result and the finding that rBCG expressing β -gal have been isolated at least 4 weeks after immunization, support our interpretation that the continuing production of antibodies is due to antigen molecules being released from the rBCG over long periods of time *in vivo*.

Because mycobacteria are rapidly phagocytosed and contained within the endosomal compartment of phagocytic

cells^{24,25}, it was not unexpected that rBCG would be an effective vehicle for inducing both T-helper cells capable of inducing B-cell production of IgG antibodies and the T cells capable of secreting lymphokines such as IFN- γ (Fig. 4b). It was not obvious, however, that it would be effective in stimulating cytotoxic T lymphocytes. That BCG can generate CTL restricted by MHC class I in mice^{26,27} encouraged us to assay for cytotoxic activity in animals immunized with rBCG. CTL were generated to both β -gal and HIV-1 gp 120 (Fig. 5). There are two possible interpretations: some rBCG may escape from lysosomes and penetrate into the cytoplasmic compartment; or because the general examples of antigen processing come mainly from studies of antigens that persist for only short times in antigen-presenting cells, conceivably antigens produced by rBCG persisting for long times in the endosomal compartment can be selected and presented to T cells both by MHC class I and class II antigens.

Although the immune responses obtained to recombinant antigens expressed in BCG reported here are encouraging, much remains to be learned before rBCG can be perfected as a multivaccine vehicle. Conditions may have to be adjusted to allow optimal expression of different antigens. For some it may be important to include signal sequences that enable recombinant proteins to be secreted intact from the rBCG. New formulations for maximizing the effectiveness of rBCG given as an oral vaccine remain to be developed. A major challenge will

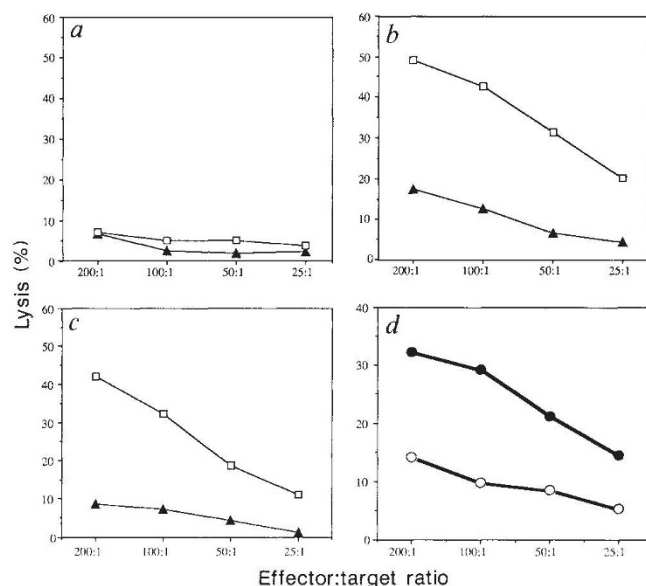


FIG. 5 Cytotoxic T-lymphocyte responses to recombinant antigens expressed in rBCG. a–c, Specific cytotoxicity to target cells expressing β -galactosidase. BALB/c mice were immunized as indicated. Splenocytes (ACK-treated, $5 \times 10^6 \text{ ml}^{-1}$) were stimulated *in vitro* in 10 ml in upright T25 flasks by coculture for 5 days with mitomycin C-treated cells transfected with the lacZ gene (C3–4 cells)³⁵ ($5 \times 10^5 \text{ ml}^{-1}$). Then a 4 h ^{51}Cr -release assay was done using P815 (\blacktriangle) and P13.1 cells³⁶ (P815 cells transfected with the lacZ gene) (\square) as targets. Effector:target ratios are as indicated using 5,000 targets per well. Specific lysis was calculated as follows: % specific lysis = $100 \times (\text{release by effector cells} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$. Spontaneous release in the absence of effector cells was less than 20% of maximal release by 10% SDS in all cases. a, β -gal-IFA ($10 \mu\text{g i.p.}$); b, rBCG::lacZ ($2 \times 10^6 \text{ c.f.u. i.p.}$); c, rBCG::lacZ ($2 \times 10^2 \text{ c.f.u. i.v.}$). d, Specific cytotoxicity to the HIV-1 envelope immunodominant CTL epitope (P18 peptide). BALB/c mice were immunized with 10^6 c.f.u. rBCG containing the integrating vector expressing gp 120 (pMV361::gp 120). Splenocytes were cocultured as above with mitomycin C-treated P815 cells ($5 \times 10^5 \text{ ml}^{-1}$) that were pulsed with $250 \mu\text{g ml}^{-1}$ of P18 peptide for 1 h. After incubation for 5 days, cells were assayed for killing by 4 h ^{51}Cr -release as above using P815 targets with (\bullet) or without (\circ) pulsing for 1 h with $250 \mu\text{g ml}^{-1}$ P18 peptide.

be to immunize against antigens, particularly viral antigens, whose protective epitopes require conformations or modification, such as glycosylation and assembly, that cannot be acquired in most procaryotes. There is a precedent in viral systems, however, that priming for immunological memory at the level of the T-helper cell, as by immunization with influenza virus internal antigens, is sufficient to allow induction of accelerated antibody responses to the surface antigens upon infection with virus²⁸⁻³⁰. Thus, even if viral antigens expressed in rBCG fail to induce virus-neutralizing antibodies, immunological

memory may be generated such that an efficient secondary response would be obtained following natural infection by virus. This would be particularly important as BCG, which is unaffected by maternal antibodies, can be given earlier in life than most viral vaccines. The hope would be that T-cell memory can ensure protection against severe morbidity and death. We believe these results establish the molecular and immunological foundation for a novel live-vaccine vehicle that may prove useful in stimulating both humoral and cellular immune responses to a wide variety of viral, bacterial and protozoal antigens. □

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LETTERS TO NATURE

Giant tunnelling anisotropy in the high- T_c superconductor $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$

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TUNNELLING spectroscopy has been one of the most fruitful methods in the study of superconductors^{1,2}. Excellent agreement has been obtained between theory and experiment, and even the fine details of the tunnelling spectra for conventional, low-transition-temperature (low- T_c) superconductors have been explained in terms of electron-phonon interactions. The low- T_c materials are generally isotropic enough for accurate measurements to be made on polycrystalline specimens; in contrast, it has been difficult to obtain reliable and reproducible tunnelling data for the highly anisotropic high- T_c materials³. We have overcome these difficulties by performing break-junction tunnelling measurements⁴ on extremely thin single crystals, and show here that the tunnelling spectra of $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ are indeed highly anisotropic. In the superconducting state, for electrons tunnelling parallel to the copper oxide planes, there are no electronic states at the Fermi level. In the normal state the tunnelling conductance is nearly independent of the d.c. bias voltage. Tunnelling perpendicular to the copper oxide planes was found to be qualitatively different from that parallel to the planes, and we suggest that electron scattering processes play an important role here.

In its most ideal realization, an electron tunnelling measurement provides us with the electronic density of states (DOS)¹. In particular, for a superconductor-insulator-superconductor junction, one can directly measure the energy gap 2Δ by observing a sudden increase of conductivity at bias voltages, V , exceeding $2\Delta/e$ (here e is the electron charge). The bias-voltage-dependent tunnelling conductance (the 'tunnelling spectrum') is related to the energy-dependent DOS by a simple integral¹. At low temperatures the conductivity of the junction is close to zero for $V < 2\Delta/e$, indicating zero DOS. For most of the low- T_c superconductors the experiments are very well described by BCS theory⁵. The copper-oxide-based high- T_c superconductors, however, did not exhibit the expected zero conductance^{5,7}. One is forced to assume either that BCS theory is not valid for these materials, or that the electron transport cannot be described as an ideal tunnelling process. Is it possible, for example, that the tunnelling is strongly influenced by inelastic electron scattering? Complex tunnelling conductance curves and 'zero-bias anomalies' have been seen in junctions with magnetic impurities⁸⁻¹³. The well known structural anisotropy of the copper-oxide-based high- T_c superconductors may also result in nontrivial effects. The tunnelling process is inherently anisotropic, because the particles with non-perpendicular incidence angle are less likely to travel through the potential barrier. What happens if the Fermi surface of the material is so anisotropic that there are no electron momenta perpendicular to the junction surface?

We attempted to answer these questions by performing tunnelling measurements on break junctions⁴ fabricated at low temperature, in vacuum. By using ultra-thin single crystals of $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$, we were able to obtain reproducible superconductor-insulator-superconductor tunnelling spectra in high- T_c superconductors. We found that parallel and perpendicular to

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