CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Nov. 1996, p. 761–768 Vol. 3, No. 6
1071-412X/96/$04.00+0

A Novel Method for Monitoring *Mycobacterium bovis* BCG Trafficking with Recombinant BCG Expressing Green Fluorescent Protein

YI LUO, AKOS SZILVASI, XIAOHONG CHEN, WILLIAM C. DEWOLF, AND MICHAEL A. O'DONNELL*

Division of Urology, Beth Israel Hospital, Harvard Medical School, Boston, Massachusetts 02215

Received 7 May 1996/Accepted 23 July 1996

To better understand intracellular and extracellular trafficking of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) when used as an intravesical agent in the treatment of transitional cell carcinoma (TCC) of the bladder, recombinant BCG (rBCG) expressing the jellyfish green fluorescent protein (GFP) was created. When the MB49.1 murine TCC cell line was incubated with GFP-expressing rBCG, internalization of the pathogen could be directly visualized by UV microscopy and quantitated by flow cytometry. The in vitro internalization of the GFP rBCG by the bladder tumor cells was temperature dependent, occurring most readily at 37°C and being severely inhibited at 4°C. Optimum internalization was achieved in vitro at a 10:1 BCG-to-tumor cell ratio over 24 h during which approximately 16% of the tumor cells became infected. Cytochalasin B, a phagocytosis inhibitor, abrogated the ingestion by almost 100% at a concentration of 200 μg/ml, indicating that contractile microfilaments likely played an important role in this process. By using mitomycin, a DNA cross-linking reagent, to inhibit proliferation of MB49.1 cells, clearance of about 40% of the green rBCG was achieved by 3 days postinfection. No significant difference between the GFP rBCG and wild-type BCG was observed in the ability to induce the expression of cell membrane proteins of major histocompatibility classes I and II, ICAM-I and -II, B7-1 and -2, or Fas from MB49.1 cells or cytokine production from mouse spleen cells. These results indicate that GFP rBCG may serve as a useful substitute for wild-type BCG in future studies of in vivo trafficking during experimental and clinical immunotherapy.

The successful application of live *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) as an intravesical agent in the treatment of superficial transitional cell carcinoma (TCC) of the bladder has been well documented and accepted (21, 26, 30). Although extensive studies have suggested that intravesical BCG acts upon TCC by inducing a complex localized immune response leading to the destruction of bladder cancer, the exact mechanism by which BCG mediates the antitumor activity remains unclear. It has been proposed that there is a two-phase process for BCG action, an initiation phase and an effector phase (37). During the initiation phase, this intracellular pathogen attaches to the bladder epithelium and is subsequently phagocytosed by macrophages and bladder epithelial cells (4, 9, 19, 20, 39). Once ingested, BCG is processed by the epithelial cells, resulting in BCG-derived antigens being expressed on their cell surface for immune recognition (22, 31). During the effector phase, multiple cellular compartments are activated, including macrophages, NK cells, B cells, $CD4^+$ T cells, $CD8^+$ T cells, and γδ T cells (6, 16, 18, 27, 33, 47, 49, 53). The release of cytokines by these activated immune cells and infected bladder epithelial cells may augment the immune response, resulting in the destruction of bladder cancer.

Understanding of the trafficking of intravesical BCG during the treatment of TCC is important for exploring the mechanisms by which this intracellular pathogen mediates antitumor activity. To date, little is known about the fate of intravesical BCG after intraluminal attachment. By electron microscopy, both human (T-24) and mouse (MBT-2) transitional epithelial cells were demonstrated to be capable of internalizing BCG in vitro (4, 20). Internalized and degraded BCG organisms in urothelial cells from bladder washes of intravesical BCG-treated patients were also identified by electron microscopic observation (4). By use of fluorescence microscopy and fluorescein isothiocyanate (FITC)-labeled BCG, the ability of T-24 cells to take up the pathogen was also observed (20). Although both electron microscopy and FITC labeling are useful approaches to the internalization study, they require either a tedious and time-consuming sampling process or treatment of the pathogen prior to infection. In order to provide a better approach to monitoring BCG trafficking, we initiated the present study to genetically engineer novel BCG strains to efficiently express green fluorescent protein (GFP), an in vivo real-time marker molecule from the jellyfish *Aequorea victoria* (32). We demonstrated that the green recombinant BCG (rBCG) organisms were clearly visualizable by UV microscopy, and the internalization of the rBCG organisms by murine TCC MB49.1 cells was quantitatively measurable by flow cytometry. Several factors affecting phagocytosis of BCG by TCC cells were also determined. Comparison of the green rBCG and its parental form for their immunostimulatory properties showed no difference, suggesting that the green rBCG may serve as a useful substitute for wild-type BCG for monitoring BCG traffic in vitro and in vivo.

MATERIALS AND METHODS

Plasmids and bacterial strains. The previously described *Escherichia coli*-BCG shuttle vector pMOD-4 (29) was modified to create a dual-promoter expression plasmid, pMOD-12, by replacing the rat interleukin 2 (IL-2) coding sequence with the mycobacterial heat shock protein 70 (hsp70) promoter (gift of Anna Aldovini) (1) flanked by two disparate multiple cloning sites (Fig. 1A). The wild-type GFP (wtGFP) cDNA-containing plasmid (pGFP) was purchased from Clontech (17, 32). The Ser-65–Thr mutant GFP (mutGFP) cDNA-containing plasmid (pRSET B) was kindly provided by R. Y. Tsien (13).

E. coli competent cells (XL1-Blue MR) were obtained from Stratagene. *M. bovis* BCG (Pasteur) was obtained through the American Type Culture Collection and grown in 7H9 Middlebrook medium (Difco) supplemented with 10%

* Corresponding author. Mailing address: Division of Urology, Beth Israel Hospital, 330 Brookline Ave., Boston, MA 02215. Phone: (617) 667-4075. Fax: (617) 667-7292.

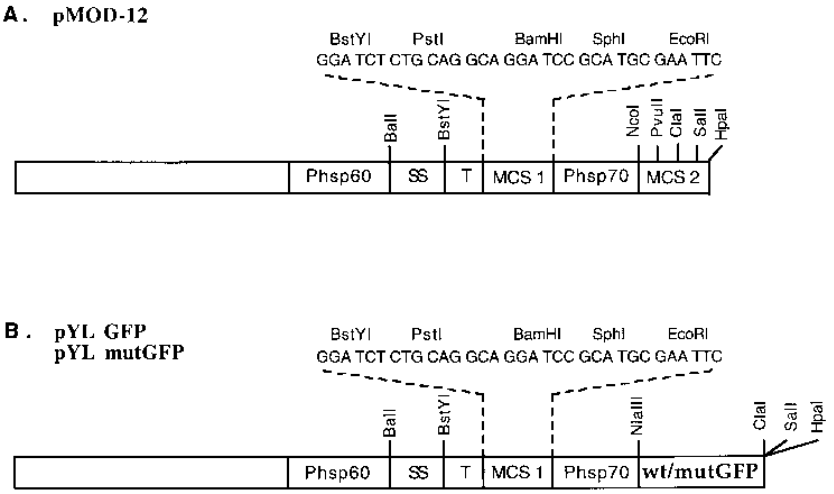


FIG. 1. Schematic illustrations of the parental *E. coli*-BCG shuttle vector pMOD-12 (A) and constructed GFP-containing plasmids pYL GFP and pYL mutGFP (B). SS, BCG α-antigen signal sequence; T, influenza virus hemagglutinin epitope tag sequence; MCS, multiple cloning site. The illustrations are not to scale.

ADC (5% bovine serum albumin fraction V, 2% dextrose, and 0.85% NaCl) and 0.05% Tween 80 (Sigma).

Construction of expression plasmids and rBCG strains. A schematic representation of the plasmids constructed for GFP expression is shown in Fig. 1B. Using the appropriate template plasmid for wtGFP or mutGFP, one pair of oligonucleotide primers was utilized in the PCR amplifications to produce cDNA inserts for cloning into the *Nco*I-*Cla*I sites of pMOD-12. The sequence of the upstream primer was CGTCAGtcatgaTGAGTAAAGGAGAAGAACTTTTC (*Bsp*HI site in lowercase), and that of the downstream primer was CGTCatcga tCTATTTGTATAGTTCATCCATGC (*Cla*I site in lowercase). The upstream primer overlapped the first one to eight codons of GFP-encoding sequences, whereas the downstream primer terminated at the stop codon UAG of the sequence.

The GFP plasmids (pYL GFP and pYL mutGFP) were constructed by ligating the *Bsp*HI-*Cla*I-digested cDNA inserts for wtGFP and mutGFP into the *Nco*I-*Cla*I site of pMOD-12, resulting in a new *Nla*III restriction site. All DNA manipulations followed previously described procedures (40). *E. coli* XL1-Blue MR cells were transformed with the GFP-containing *E. coli*-BCG shuttle plasmids according to the manufacturer's instructions and selected on kanamycin (30 μg/ml) Luria-Bertani agar plates. The *E. coli*-derived plasmids with correct structures verified by restriction analysis were then transformed into BCG by electroporation as described previously (44). The transformed BCG cells were plated on Middlebrook 7H10 Bacto agar (Difco) supplemented with 10% ADC and 30 μg of kanamycin per ml. Individual colonies showing bright green fluorescence under illumination from a long-wavelength UV lamp were picked and grown in BCG culture medium for the internalization studies.

Internalization assay. A cloned subline (MB49.1) of the parental murine TCC cell line MB49 (gift of Tim Ratliff, St. Louis, Mo.) was used for the in vitro studies. Cells were grown in flat-bottomed six-well tissue culture plates (Nunc) containing RPMI 1640 medium (Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum and 30 μg of kanamycin per ml. When the cell density reached approximately 70% confluence, the monolayer cells were washed once with culture medium, and 1.5×10^7 CFU of GFP-expressing rBCG in 2 ml of the culture medium were added to each well. The cell mixtures were incubated for various times at 37°C in a humidified 5% CO_2 incubator, washed four times with culture medium to eliminate extracellular bacteria, removed from the plates with 0.02% EDTA solution, and resuspended in the culture medium. For more-stringent removal of extracellular bacteria, a further step with Ficoll-Paque (Pharmacia) density centrifugation was applied to the washed infected MB49.1 cells. Previous studies in our laboratory using the vital dye indicator MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Thiazolyl blue; Sigma) have shown that this technique eliminates >99.9% of suspended BCG bacteria. The cells collected from the interface layer after centrifugation were used for characterization. Internalization of the green rBCG bacteria by MB49.1 cells was scored by visual inspection with a Nikon Microphot-SA fluorescence microscope and quantitated by cell counting using the same fluorescence microscope or by flow cytometry using a FACScan flow cytometer (Becton Dickinson) with CellQuest software. An appropriate window was set for the analysis to exclude any free extracellular green rBCG organisms from those associated with MB49.1 cells. For inhibition studies, the above protocol for internalization was performed either at 4°C or in the presence of cytochalasin B (Sigma) at a titration dose ranging from 25 to 400 μg/ml. For the clearance study, mitomycin (Sigma) was added to a final concentration of 50 μg/ml, and the mixture was incubated at 37°C for 20 min and then washed four times.

Cytokine production by BCG-infected mouse spleen cells and TCC cells. The spleens were removed aseptically from 8- to 12-week-old female C57BL/6 mice (Jackson Labs, Bar Harbor, Maine) and mechanically dispersed. Erythrocytes in the pooled spleen cell collection were lyzed with ACK lysing buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA; pH 7.4), washed with RPMI 1640 medium, and grown in flat-bottomed 24-well tissue culture plates (Nunc) at a density of 4×10^6 cells per well containing 1 ml of RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 30 μg of kanamycin. The splenocytes were infected with a series of different BCG doses of either wild-type BCG, MV261 BCG (a control BCG transformed with the nonexpressing plasmid pMV261 [46]), or mutGFP rBCG and incubated at 37°C for 3 days in a humidified CO_2 incubator. The supernatants were then harvested, and cytokine profiles were determined by sandwich enzyme-linked immunosorbent assays (ELISAs) using appropriate paired antibodies according to the instructions of the manufacturer (PharMingen).

A cytokine profile for the culture supernatants of a mouse TCC cell line, MB49.1, was also determined by ELISAs after infection with 1.5×10^7 CFU of either MV261 or mutGFP rBCG and incubation at 37°C for 2 days as mentioned above.

Phenotypic analysis of mouse TCC MB49.1 cells infected with BCG. MB49.1 cells were incubated with 1.5×10^7 CFU of either MV261 or mutGFP rBCG at 37°C for 2 days, washed four times with RPMI 1640 medium, and removed from the tissue culture plates for cell staining and analysis. As a positive control, MB49.1 cells were cultured in the presence of recombinant murine gamma interferon (IFN-γ) (24 ng/ml), recombinant murine tumor necrosis factor alpha (TNF-α) (5 ng/ml), or both. A panel of fluoresceinated monoclonal antibodies recognizing murine cell surface markers was added at appropriate concentrations to aliquots of 10^6 cells according to the instructions of the manufacturer (PharMingen). After incubation on ice for 30 min, the cells were washed in ice-cold phosphate-buffered saline containing 0.01% sodium azide and analyzed by flow cytometry.

RESULTS

Construction and characterization of BCG recombinants expressing GFP. To express wtGFP and mutGFP in BCG, two plasmids (pYL GFP and pYL mutGFP) were constructed as described in Materials and Methods and as shown in Fig. 1B. In these plasmids, the expression of wtGFP and mutGFP was driven by the hsp70 promoter, leaving an upstream expression cassette available as a second gene cloning site. The upstream expression cassette was composed of a BCG hsp60 promoter sequence, a BCG α-antigen signal sequence, an influenza virus hemagglutinin epitope tag sequence, and a multiple cloning site. These sequences facilitated the transcription, secretion, and detection of a putative gene inserted in the multiple clon-

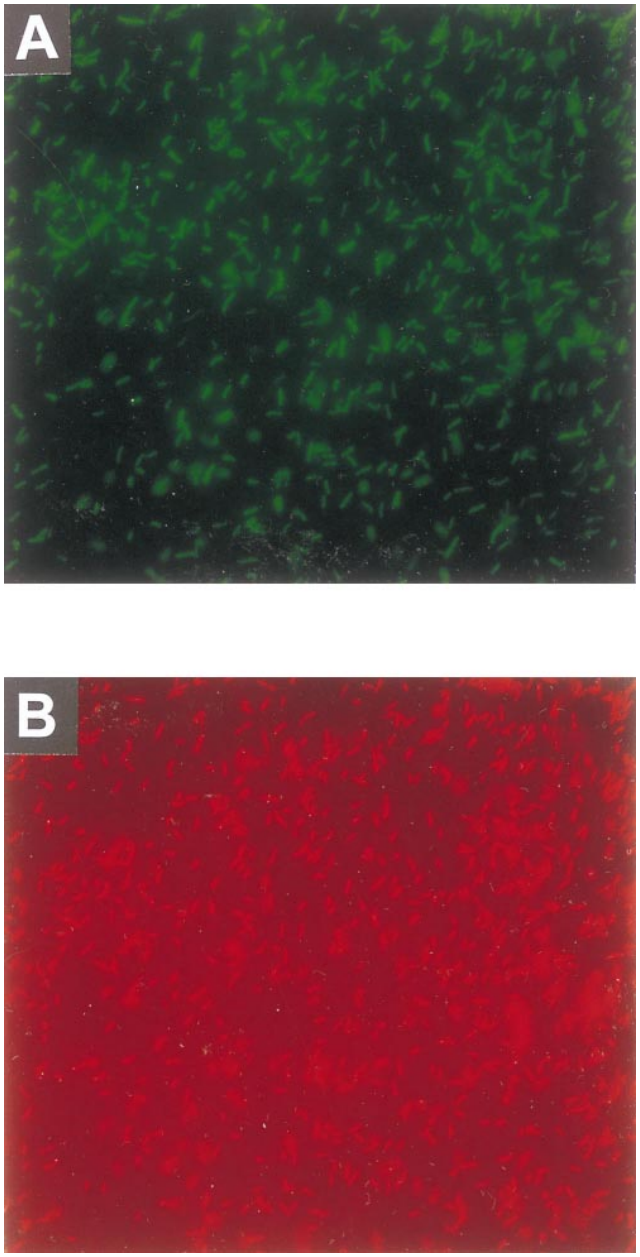


FIG. 2. Expression of mutGFP in BCG. BCG Pasteur strain was transformed with mutGFP-containing plasmid. The culture of the green rBCG strain was photographed under a fluorescence microscope with a 100× oil immersion objective and a filter set suitable for fluorescein detection (A). The photograph of the same culture stained with ethidium bromide (50 μg/ml) was taken as described above (B).

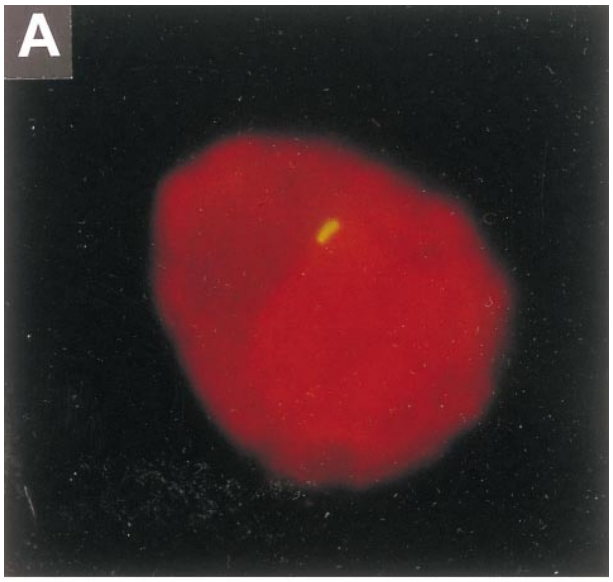
ing site. This expression cassette has been successfully used for the functional expression of mouse IL-2 along with the simultaneous expression of wtGFP driven by the hsp70 promoter (data not shown).

wtGFP and mutGFP rBCG strains were generated by transformation of the BCG Pasteur strain with the respective plasmids by electroporation. As shown in Fig. 2A, individual mutGFP-transformed BCG bacilli are clearly and uniformly imaged under UV illumination with a fluorescence microscope. BCG bacilli transformed with wtGFP were also readily visible but with a weaker intensity (not shown). Quantitation of the ratio between emission fluorescence at 510 nm versus optical density at 600 nm (a measure of BCG concentration) for the two GFP BCG strains revealed a four- to fivefold increase in favor of mutGFP rBCG. The fluorescence expressed by either green rBCG strain is stable, and no apparent photobleaching has been observed even after storage for 1 year at 37°C. The doubling times of both rBCG strains were observed to be the same as those for other rBCG strains transformed with IL-2-expressing plasmids (29). Since the spectral property of mutGFP is more suitable than that of wtGFP for standard fluorescence microscopy as well as flow cytometry (13), the mutGFP rBCG strain was used for most of the present studies.

GFP-expressing rBCG organisms are internalized by the mouse TCC cell line MB49.1 in an energy- and microfilament-dependent process. As shown in Fig. 3, the mutGFP rBCG organisms internalized by MB49.1 cells can be easily determined by morphological recognition under a fluorescence microscope. These internalized bacteria are restricted to the cytoplasmic compartment of the tumor cells, in either a single bacillary form or an accumulated clumped form. The latter could result from uncontrolled proliferation of the intracellular bacteria or from ingestion of a single aggregate. To discriminate extracellular bacteria that attached to the cell membrane from those which were truly internalized, ethidium bromide was added to the cell suspension. Because transit of ethidium bromide across cell membranes is greatly retarded relative to uptake across bacterial cell walls (12), extracellular BCG organisms fluoresce red and quench GFP fluorescence (Fig. 2B), while internalized organisms remain green. After four washes, red bacteria associated with MB49.1 cells were rarely seen, indicating that the bacteria were not tightly bound to the cell membrane and could easily be removed by washing. Quantitation of BCG internalization was performed by flow cytometric analysis and conformed closely to that determined by fluorescence microscopic counting. The internalization occurred in a time-dependent manner, and the maximum internalization (approximately 16%) was observed at 16 h during the 24-h evaluation period (Fig. 4). Longer incubation periods inconsistently resulted in higher or lower values for internalization, possibly because of direct toxic effects of bacterial products on the tumor cells (data not shown) (35, 36).

To determine whether the internalization process was dependent on membrane movement and vesicular traffic, internalization of mutGFP rBCG bacteria cultured at different ratios with MB49.1 cells at 4 versus 37°C was compared. Consistent with previous reports for other systems (4), the low temperature significantly inhibited the internalization process (Fig. 5). The optimum internalization was observed at a rBCG dose of around 0.125 optical density at 600 nm units per ml ($\sim 4 \times 10^6$ CFU/ml). Compared with the number of tumor cells present, this was calculated to be about a 10:1 rBCG-to-tumor cell ratio. The apparent reduction in internalization that occurred at greater rBCG concentrations is not known but could similarly represent the effect of toxic bacterial by-products on tumor cell viability (35, 36). Internalization of wtGFP rBCG by MB49.1 cells showed a pattern similar to that of mutGFP rBCG (data not shown). These observations were reproducible in at least two separate experiments. Similar to the low-temperature effect on internalization, cytochalasin B, an agent known to disrupt microfilament assembly (2), inhibited internalization by close to 100% at a concentration of 200 μg/ml (data not shown). The inhibitory effects of both low temperature and cytochalasin B were morphologically confirmed by microscopic observation.

Clearance of ingested BCG organisms. To test the intracellular duration of the ingested green rBCG bacteria in mouse



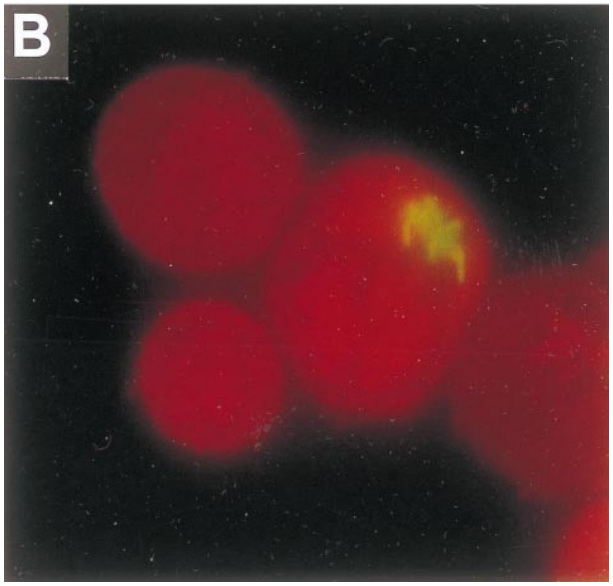


FIG. 3. Internalization of green rBCG organisms by mouse TCC MB49.1 cells. Tumor cells were incubated with 1.5×10^7 CFU of mutGFP rBCG per well for 18 h at 37°C. After four washes, the infected cells were stained with ethidium bromide (50 μg/ml) and photographed as described for Fig. 2 but with double exposures using appropriate filters.

TCC cells, a clearance assay was performed. Mitomycin, a DNA cross-linking reagent, was applied to MB49.1 cells to eliminate possible dilution resulting from cellular proliferation. Trypan blue staining of the cells after mitomycin treatment revealed no loss in cell viability. The internalized rBCG organisms were observed to gradually clear out from MB49.1 cells by approximately 40% over a 3-day observation period (Fig. 6).

GFP rBCG retains its ability to induce cytokine expression and phenotypic alterations from splenocytes and tumor cells. To test any possible alterations in its immunostimulatory properties resulting from GFP gene expression in rBCG, the cytokine profiles of culture supernatants from mouse spleen cells and TCC MB49.1 cells infected with wild-type BCG, MV261 BCG, or mutGFP rBCG were compared. In Table 1, cytokine production by splenocytes infected with 2.4×10^6 CFU of BCG per ml is shown as an example. Although some variations in the level of cytokine production were observed among the groups, they were not statistically significant. All three types of BCG strains efficiently stimulated the splenocytes to express cytokines, with IFN-γ, TNF-α, and IL-6 dominating. In contrast, the ability of BCG to directly induce cytokine expression from MB49.1 cells was limited. Only granulocyte-macrophage

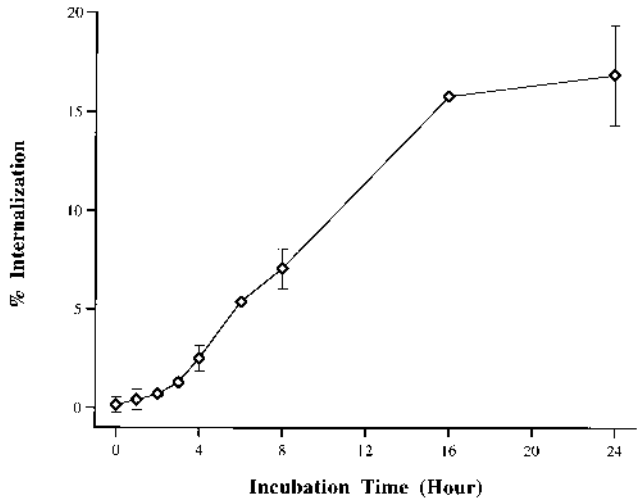


FIG. 4. Time course of mutGFP rBCG internalization by mouse TCC MB49.1 cells. The tumor cells were incubated with 1.5×10^7 CFU of the green rBCG per well for up to 24 h at 37°C. The percentage of the cells containing green rBCG organisms was determined by flow cytometry and visually confirmed by fluorescence microscopy.

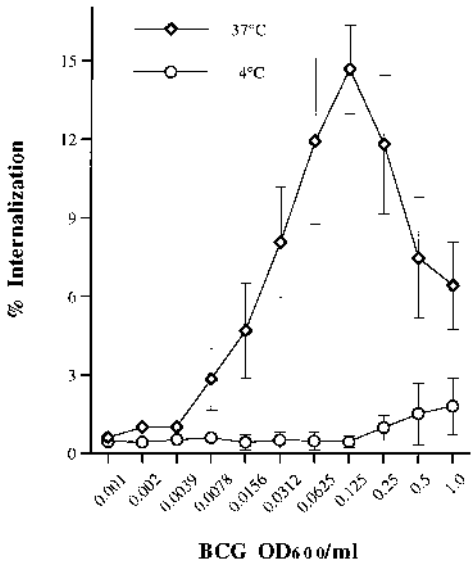


FIG. 5. Effect of temperature on internalization of mutGFP rBCG organisms in mouse TCC MB49.1 cells. The tumor cells were incubated with serial twofold dilutions of the green rBCG organisms for 18 h at 4 or 37°C. The percentage of the cells containing green rBCG bacteria was determined by flow cytometry and visually confirmed by fluorescence microscopy. OD_{600}, optical density at 600 nm.

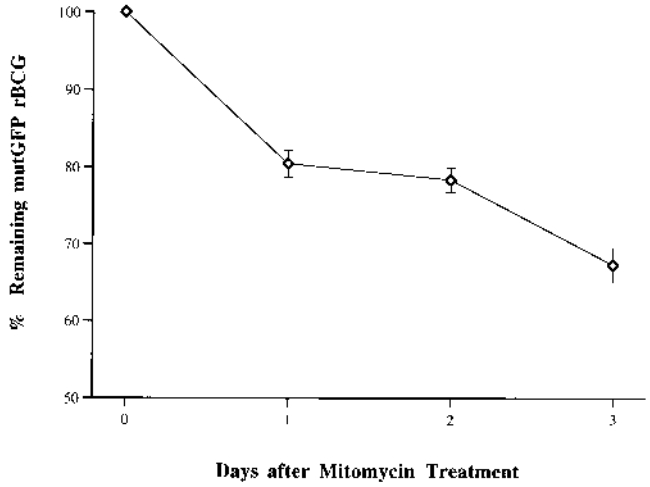


FIG. 6. Intracellular duration of ingested mutGFP rBCG bacteria in mouse TCC MB49.1 cells. The tumor cells were first incubated with 1.5×10^7 CFU of the green rBCG per well for 18 h at 37°C. After a washing, the infected tumor cells were treated with mitomycin for 20 min and the incubation was continued at 37°C. Infected cells were then collected at 0, 1, 2, or 3 days after mitomycin treatment for flow cytometry analysis.

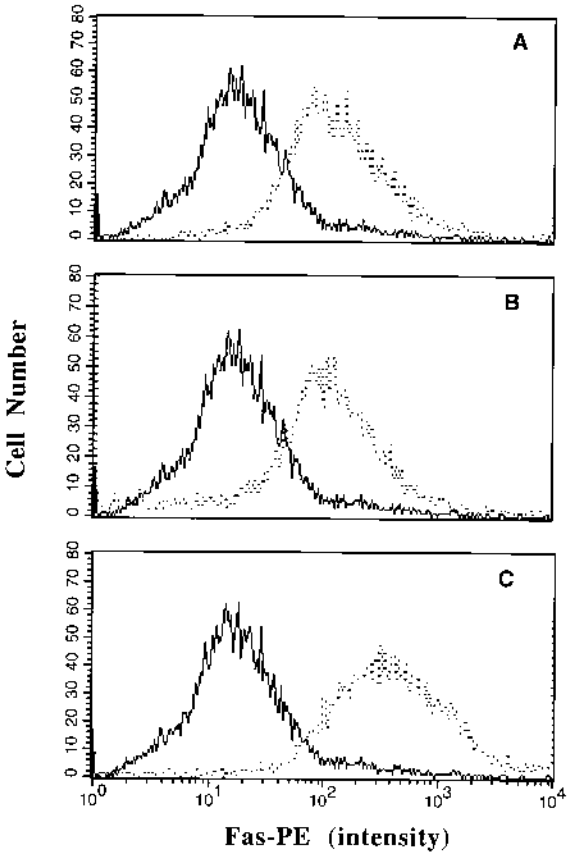


FIG. 7. Fas expression of BCG-infected mouse TCC MB49.1 cells as determined by flow cytometry. Tumor cells were incubated with 1.5×10^7 CFU of either MV261 BCG (A) or mutGFP rBCG (B) per well for 2 days at 37°C. As a positive control, cells were incubated with TNF-α (5 ng/ml) (C). An excess of phosphatidylethanolamine (PE)-labeled anti-Fas monoclonal antibody (PharMingen clone JO-2) was used, and the individual-cell-associated fluorescence was measured by flow cytometry. Basal Fas expression of nontreated MB49.1 cells is also shown (solid lines).

colony-stimulating factor (GM-CSF) expression was significantly upregulated.

Phenotypic analysis of the BCG-infected MB49.1 cells was also carried out to compare the effect of mutGFP rBCG infection with that of infection by MV261 BCG, an rBCG transformed with the nonproducer plasmid pMV261 (46). No apparent difference between these two BCG strains in their ability to change the expression of surface molecules of infected MB49.1 cells in vitro was observed. BCG infection did not directly lead to heightened expression of major histocompatibility (MHC) classes I and II, ICAM-I and -II, or B7-1 and -2 (data not shown). However, Fas expression of MB49.1 cells was substantially upregulated by the infection with either BCG strain (Fig. 7 and 8). This elevated level of expression of Fas molecules on the MB49.1 cell surface, however, was not dependent on BCG phagocytosis, as cells which internalized GFP rBCG organisms were upregulated to a degree similar to that of cells that did not (Fig. 8B, plot c).

DISCUSSION

We have constructed *M. bovis* BCG recombinants expressing jellyfish GFP, which causes the whole organism to fluoresce under a defined wavelength of UV illumination. The GFP expressed by the rBCG strains is quite stable and does not show apparent toxicity to the organism. Studies with culture cells in vitro have verified that the GFP-expressing rBCG strains retain the same immunostimulatory property as the parental BCG strains with respect to the direct effect on tumor cells or induction of cytokines by immune cells. We have fur-

TABLE 1. Cytokine production by BCG-infected cells

Cell type and BCG used	Cytokine production (pg/ml)[a]						
	IL-2	IL-6	IL-10	IL-12	GM-CSF	IFN-γ	TNF-α
Splenocytes							
No BCG	42 ± 15	200 ± 9	65 ± 5	1,199 ± 32	9 ± 1	145 ± 47	12 ± 0
Wild-type BCG	25 ± 5	3,841 ± 205	219 ± 14	1,453 ± 5	407 ± 7	3,541 ± 1,475	1,886 ± 70
MV261 BCG	49 ± 17	3,644 ± 694	169 ± 5	1,072 ± 2	422 ± 5	1,503 ± 500	2,222 ± 30
mutGFP rBCG	96 ± 2	3,930 ± 163	292 ± 4	1,677 ± 127	281 ± 5	2,806 ± 470	2,412 ± 9
MB49.1 cells							
No BCG	0	34 ± 16	9 ± 9	84 ± 3	51 ± 1	0	0
MV261 BCG	0	28 ± 20	0	132 ± 1	3,405 ± 55	0	0
mutGFP rBCG	0	15 ± 2	16 ± 4	325 ± 2	4,082 ± 15	0	0

[a] Cytokine production was measured by ELISA. Values are means ± standard deviations.

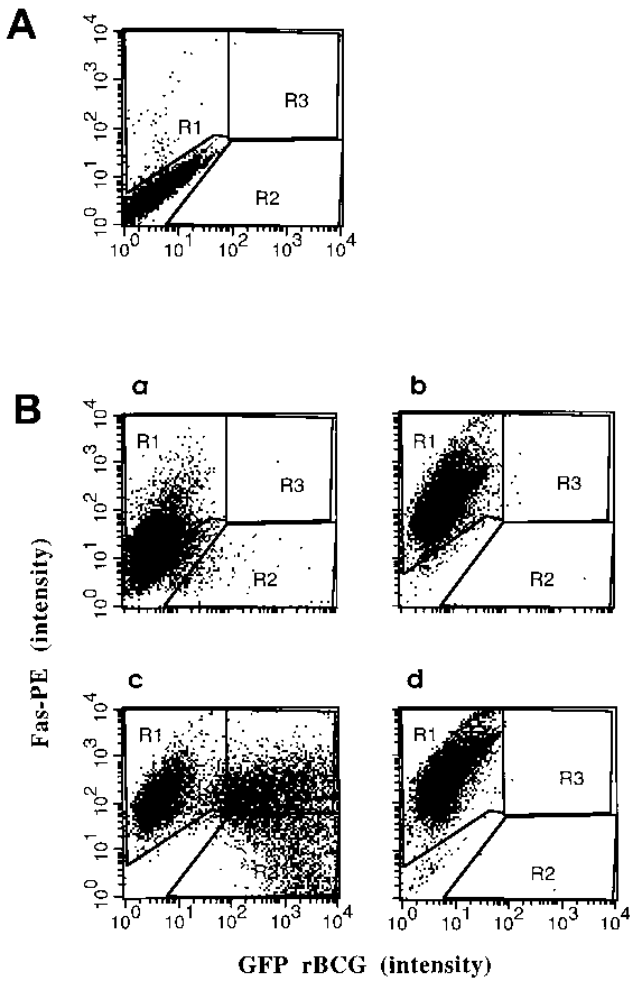


FIG. 8. Dot plots of Fig. 7. (A) Isotype control antibody used for setting gates. (B) Basal Fas expression of nontreated MB49.1 cells (a) and effects of MV261 BCG (b), mutGFP rBCG (c), or TNF-α (d) on MB49.1 cells. R1, MB49.1 cells stained with phosphatidylethanolamine (PE)-labeled anti-Fas monoclonal antibody; R2, MB49.1 cells which incorporated green rBCG organisms; and R3, MB49.1 cells fluorescing with both green rBCG and PE-labeled antibody.

ther applied the green rBCG to internalization studies in vitro in mouse TCC MB49.1 cells and demonstrated that GFP expressed by the green rBCG strains is a useful and sensitive marker for monitoring BCG trafficking. Taken together, these results suggest that the green rBCG can be used as a substitute for wild-type BCG for trafficking studies of the organism during infection in vitro and in vivo.

GFP, as a novel fluorescent marker protein, is attracting widespread interest because of its tremendous potential for in vivo gene expression and protein localization, cell lineage determination, and in situ monitoring of cell growth and development. GFP has been expressed and shown to fluoresce in a variety of prokaryotes, eukaryotes, invertebrates, vertebrates, and plants. Among the microbes, a number of organisms have been observed to express GFP, including tobacco mosaic tobamovirus (14), potato virus X (3), *Dictyostelium* spp. (15, 25), *E. coli* (8, 17), *Saccharomyces cerevisiae* (23, 41), and *Schizosaccharomyces pombe* (28). The present study has extended this observation to include *M. bovis* BCG as a host for GFP expression.

The use of GFP-expressing rBCG strains to follow pathogen trafficking during infection provides clear advantages over more cumbersome approaches such as electron microscopy and fluorophore labeling (4, 20, 42). In brief, first, the ready-to-use green pathogens can be directly applied to the target without any prior treatment process. Second, expression of fluorescence by the green rBCG strains is uniform and consistent, which makes it possible to achieve a more accurate and reliable quantitative analysis for phagocytosis of the pathogen by the target. Third, GFP is exceptionally stable and its fluorescence signal is highly resistant to photobleaching even under the high-intensity illumination of a fluorescence microscope (50). Fourth, expression of GFP fluorescence has been observed from both live and fixed cells (7), which is especially useful for in vivo monitoring of BCG trafficking. Finally, the spectral property of GFP is similar to that of FITC, which allows visualization of the green organism with standard fluorescence microscopy and quantitation with flow cytometry.

The demonstration of internalization of green rBCG organisms by mouse TCC MB49.1 cells is comparable to that previously described for internalization of wild-type BCG by both human bladder tumor cell line T-24 and mouse bladder tumor cell line MBT-2 (4). Thus, it appears that malignant urothelial cells are clearly capable of ingesting *M. bovis* BCG. The internalization process in the present study was significantly inhibited by both incubation at 4°C and treatment with cytochalasin B, conditions known to inhibit phagocytosis. This suggests that the phagocytic process requires normal microfilament function and is energy dependent. The kinetics of internalization of the green rBCG organisms by MB49.1 cells, however, differed from that described for wild-type BCG phagocytosis by both T-24 and MBT-2 cells in that maximal internalization was observed at 16 h in this study but at 3 h in the previous studies. Moreover, the capacity for internalization by MB49.1 cells observed in the present study was higher than that reported for T-24 or MBT-2 cells with internalization assays performed under similar conditions. These differences could be attributed to the different origins of the cell lines. T-24 is a human TCC line and MBT-2 is a nitrosamine *N*-[4-(5-nitro-2-furyl)-2-thiazoyl]-formamide (FANFT)-induced murine TCC of C3H/HeJ origin, while MB49 was derived from a C57BL/6 mouse after treatment with the polycyclic aromatic hydrocarbon 7,12-dimethylbenz(a)anthracene (45, 52). Cells with different genetic backgrounds could conceivably have different quantities of BCG-binding receptors expressed on their surfaces, different capacities for ingestion and intracellular clearance of the organism, or different energy requirements for phagocytosis.

Although bladder tumor cells have been clearly demonstrated to actively internalize BCG, it remains unclear whether phagocytosis is directly associated with antitumor activity. Whether the specific immune destruction of the tumor cells requires the recognition of cell surface BCG-derived antigens associated with MHC molecules by immune cytotoxic killer cells is itself unknown. Phenotypic analysis of mouse TCC MB49.1 cells infected with BCG in vitro did not show direct evidence that phagocytosis could augment tumor cell expression of MHC classes I and II, ICAM-I and -II, or B7-1 and -2, the molecules which mediate immune recognition by $CD4^+$ T cells and $CD8^+$ cytotoxic T lymphocytes. While the expression of Fas, an apoptotic mediator, was upregulated by BCG infection, the elevated level of Fas expression was observed to be related only to BCG exposure and not to BCG phagocytosis. Thus, among the limited parameters analyzed, phagocytosis of BCG organisms does not seem to be required for specific membrane-associated changes by tumor cells. Instead, other indirect processes may be responsible for tumor cell immune recognition. The local production of cytokines such as IFN-γ

and TNF-α induced from immune competent cells by BCG, for instance, has been demonstrated to be capable of upregulating the expression of MHC class I and II antigens as well as adhesion molecules on bladder tumor cells (33). These same cytokines have also been observed to augment the expression of Fas (24, 51). The production of these cytokines is effectively induced by BCG infection in vitro as well as in urine samples from patients undergoing intravesical BCG treatment in vivo (5, 10, 29, 34, 43, 48). The ability of bladder tumor cells to produce GM-CSF upon BCG infection has also been observed and documented by others (11, 38). Whether this phenomenon is associated with phagocytosis is unknown.

We have demonstrated the application of a prototype of GFP-expressing rBCG for monitoring mycobacterial pathogen trafficking in vitro. The GFP-containing vectors described here can also be used to express and secrete various cytokines when cloned into the upstream expression cassette. Indeed, an rBCG strain simultaneously expressing both GFP and mouse IL-2 has been successfully cloned. These green rBCG strains both with and without a functioning cytokine gene provide a novel and ideal means for future in vivo trafficking studies. The same GFP plasmid vectors may also provide a means for studying the trafficking of other, more pathogenic mycobacterial organisms during infection.

ACKNOWLEDGMENT

This work was supported in part by grant CA64230-02 provided by the NCI.

REFERENCES

1. **Aldovini, A., and R. A. Young.** 1991. Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. Nature (London) **351:** 479–482.
2. **Allison, A. C., P. Davies, and S. De Petris.** 1971. Role of contractile microfilaments in macrophage movement and endocytosis. Nature (London) New Biol. **232:**153–155.
3. **Baulcombe, D. C., S. Chapman, and S. Santa Cruz.** 1995. Jellyfish green fluorescent protein as a reporter for virus infections. Plant J. **7:**1045–1053.
4. **Becich, M. J., S. Carroll, and T. L. Ratliff.** 1991. Internalization of Bacille Calmette-Guerin by bladder tumor cells. J. Urol. **145:**1316–1324.
5. **Bohle, A., C. H. Nowc, A. J. Ulmer, J. Musehold, J. Gerdes, A. G. Hofstetter, and H. D. Flad.** 1990. Elevations of cytokines interleukin-1, interleukin-2, and tumor necrosis factor in the urine of patients after intravesical bacillus Calmette-Guerin immunotherapy. J. Urol. **144:**59–64.
6. **Bohle, A., A. Thanhauser, A. Ulmer, M. Ernst, H.-D. Flad, and D. Jocham.** 1993. Dissecting the immunobiological effects of bacillus Calmette-Guerin (BCG) in vitro: evidence of a distinct BCG-activated killer (BAK) cell phenomenon. J. Urol. **150:**1932–1937.
7. **Chalfie, M.** 1995. Green fluorescent protein. Photochem. Photobiol. **62:**651–656.
8. **Chalfie, M., Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher.** 1994. Green fluorescent protein as a marker for gene expression. Science **263:**802–805.
9. **Cheng, D. L., W. P. Shu, J. C. Choi, E. J. Margolis, M. J. Droller, and B. C. Liu.** 1994. Bacillus Calmette-Guerin interacts with the carboxyl-terminal heparin binding domain of fibronectin: implications for BCG-mediated antitumor activity. J. Urol. **152:**1275–1280.
10. **De Boer, E. C., W. H. Dejong, P. A. Steerenberg, L. A. Aarden, E. Tetteroo, E. R. De Groot, A. P. M. Van der Meijden, P. D. J. Vegt, F. M. J. Debruyne, and E. J. Ruitenberg.** 1992. Induction of urinary interleukin-1 (IL-1), IL-2, IL-6 and tumor necrosis factor during intravesical immunotherapy with bacillus Calmette-Guerin in superficial bladder cancer. Cancer Immunol. Immunother. **34:**306–312.
11. **De Reijke, T. M., P. C. Vos, E. C. De Boer, R. F. Bevers, W. H. De Muinck Keizer, K. H. Kurth, and D. H. Schamhart.** 1993. Cytokine production by the human bladder carcinoma cell line T24 in the presence of bacillus Calmette-Guerin (BCG). Urol. Res. **21:**349–352.
12. **Drevets, D. A., and P. A. Campbell.** 1991. Macrophage phagocytosis: use of fluorescence microscopy to distinguish between extracellular and intracellular bacteria. J. Immunol. Methods **142:**31–38.
13. **Heim, R., A. B. Cubitt, and R. Y. Tsien.** 1995. Improved green fluorescence. Nature (London) **373:**663–664.
14. **Heinlein, M., B. L. Epel, H. S. Padgett, and R. N. Beachy.** 1995. Interaction of tobamovirus movement proteins with the plant cytoskeleton. Science **270:**1983–1985.
15. **Hodgkinson, S.** 1995. GFP in *Dictyostelium*. Trends Genet. **11:**327–328.
16. **Huygen, K., D. Abramowicz, P. Vandenbussche, F. Jacobs, J. DeBruyn, A. Kentos, A. Drowart, J. P. Van Vooren, and M. Goldman.** 1992. Spleen cell cytokine secretion in *Mycobacterium bovis* BCG-infected mice. Infect. Immun. **60:**2880–2886.
17. **Inouye, S., and F. I. Tsuji.** 1994. *Aequorea* green fluorescent protein: expression of the gene and fluorescent characteristics of the recombinant protein. FEBS Lett. **341:**277–280.
18. **Inoue, T., Y. Yoshikai, G. Matsuzaki, and K. Nomoto.** 1991. Early appearing T cells during infection with Calmette Guerin Bacillus. J. Immunol. **146:** 2754–2762.
19. **Kavoussi, L. R., E. J. Brown, J. K. Ritchey, and T. L. Ratliff.** 1990. Fibronectin-mediated Calmette-Guerin Bacillus attachment to murine bladder mucosa. Requirement for the expression of an antitumor response. J. Clin. Invest. **85:**62–67.
20. **Kuroda, K., E. J. Brown, W. B. Telle, D. G. Russell, and T. L. Ratliff.** 1993. Characterization of the internalization of bacillus Calmette-Guerin by human bladder tumor cells. J. Clin. Invest. **91:**69–76.
21. **Lamm, D. L.** 1992. Long-term results of intravesical therapy for superficial bladder cancer. Urol. Clin. N. Am. **19:**573–580.
22. **Lattime, E. C., L. G. Gomella, and P. A. McCue.** 1992. Murine bladder carcinoma cells present antigen to BCG specific $CD4^+$ T-cells. Cancer Res. **52:**4286–4290.
23. **Lim, C. R., Y. Kimata, M. Oka, K. Nomaguchi, and K. Kohno.** 1995. Thermosensitivity of green fluorescent protein fluorescence utilized to reveal novel nuclear-like compartments in a mutant nucleoporin Nsp 1. J. Biochem. **118:**13–17.
24. **Maciejewski, J., C. Selleri, S. Anderson, and N. S. Young.** 1995. Fas antigen expression on $CD34^+$ human marrow cells is induced by interferon-gamma and tumor necrosis factor alpha and potentiates cytokine-mediated hematopoetic suppression *in vitro*. Blood **85:**3183–3190.
25. **Moores, S. L., J. H. Sabry, and J. A. Spudich.** 1996. Myosin dynamics in live Dictyostelium cells. Proc. Natl. Acad. Sci. USA **93:**443–446.
26. **Morales, A., and J. C. Nickel.** 1992. Immunotherapy for superficial bladder cancer. A developmental and clinical overview. Urol. Clin. N. Am. **19:**549–556.
27. **Murray, P. J., and R. A. Young.** 1992. Stress and immunological recognition in host-pathogen interactions. J. Bacteriol. **174:**4193–4196.
28. **Nabeshima, K., H. Kurooka, M. Takeuchi, K. Kinoshita, Y. Nakaseko, and M. Yanagida.** 1995. p93dis, which is required for sister chromatid separation, is a novel microtubule and spindle pole body-associating protein phosphorylated at the Cdc2 target sites. Genes Dev. **9:**1572–1585.
29. **O'Donnell, M. A., A. Aldovini, R. B. Duda, H. Yang, A. Szilvasi, R. A. Young, and W. C. DeWolf.** 1994. Recombinant *Mycobacterium bovis* BCG secreting functional interleukin-2 enhances gamma interferon production by splenocytes. Infect. Immun. **62:**2508–2514.
30. **O'Donnell, M. A., and W. C. DeWolf.** 1995. Bacillus Calmette-Guerin immunotherapy for superficial bladder cancer: new prospects for an old warhorse. Surg. Oncol. Clin. N. Am. **4:**189–201.
31. **Paul, W. E., and R. A. Seder.** 1994. Lymphocyte responses and cytokines. Cell **76:**241–251.
32. **Prasher, D. C., V. K. Eckenrode, W. W. Ward, F. G. Prendergast, and M. J. Cormier.** 1992. Primary structure of the *Aequorea victoria* green fluorescent protein. Gene **111:**229–233.
33. **Prescott, S., K. James, T. B. Hargreave, G. D. Chisholm, and J. F. Smyth.** 1992. Intravesical Evans strain BCG therapy: quantitative immunohistochemical analysis of the immune response within the bladder wall. J. Urol. **147:**1636–1642.
34. **Prescott, S., K. James, T. B. Hargreave, G. D. Chisholm, and J. F. Smyth.** 1990. Radioimmunoassay detection of interferon-gamma in urine after intravesical Evans BCG therapy. J. Urol. **144:**1248–1251.
35. **Pryor, K., J. Goddard, D. Goldstein, P. Stricker, P. Russell, D. Golovsky, and R. Penny.** 1995. Bacillus Calmette-Guerin (BCG) enhances monocyte- and lymphocyte-mediated bladder tumor cell killing. Br. J. Cancer **71:**801–807.
36. **Pryor, K., P. Stricker, P. Russell, D. Golovsky, and R. Penny.** 1995. Antiproliferative effects of bacillus Calmette-Guerin and interferon alpha 2b on human bladder cancer cells *in vitro*. Cancer Immunol. Immunother. **41:**309–316.
37. **Ratliff, T. L.** 1992. Role of the immune response in BCG for bladder cancer. Eur. Urol. **21**(Suppl. 2)**:**17–21.
38. **Ratliff, T. L., A. Gerber, J. Ritchey, and R. Nadler.** 1994. Modulation of bladder tumor cytokine production by BCG. J. Urol. **151:**515A.
39. **Ratliff, T. L., J. O. Palmer, J. A. McGarr, and E. J. Brown.** 1987. Intravesical bacillus Calmette-Guerin therapy for murine bladder tumors: initiation of the response by fibronectin-mediated attachment of bacillus Calmette-Guerin. Cancer Res. **47:**1762–1766.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
41. **Schlenstedt, G., C. Saavedra, J. D. Loeb, C. N. Cole, and P. A. Silver.** 1995. The GFP-bound form of the yeast Ran/TC4 homologue blocks nuclear protein import and appearance of poly$(A)^+$ RNA in the cytoplasm. Proc.

Natl. Acad. Sci. USA **92:**225–229.
42. **Schorey, J. S., Q. Li, D. W. McCourt, M. Bong-Mastek, J. E. Clark-Curtiss, T. L. Ratliff, and E. J. Brown.** 1995. A *Mycobacterium leprae* gene encoding a fibronectin-binding protein is used for efficient invasion of epithelial cells and Schwann cells. Infect. Immun. **63:**2652–2657.
43. **Shin, J. S., J. H. Park, J. D. Kim, J. M. Lee, and S. J. Kim.** 1995. Induction of tumor necrosis factor-alpha (TNF-alpha) mRNA in bladders and spleens of mice after intravesical administration of bacillus Calmette-Guerin. Clin. Exp. Immunol. **100:**26–31.
44. **Snapper, S. B., L. Lugosi, A. Jekkel, R. E. Melton, Z. T. Kieser, B. R. Bloom, and W. R. Jacobs, Jr.** 1988. Lysogeny and transformation in mycobacteria: stable expression of foreign genes. Proc. Natl. Acad. Sci. USA **85:**6987–6991.
45. **Soloway, M. S.** 1977. Intravesical and systemic chemotherapy of murine bladder cancer. Cancer Res. **37:**2918–2929.
46. **Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, W. R. Jacobs, Jr., and B. R. Bloom.** 1991. New use of BCG for recombinant vaccines. Nature (London) **351:**456–460.
47. **Thanhauser, A., A. Bohle, H. D. Flad, M. Ernst, T. Mattern, and A. J. Vlmer.** 1993. Induction of bacillus Calmette-Guerin activated killer cells from human peripheral blood mononuclear cells against human bladder carcinoma cell lines *in vitro*. Cancer Immunol. Immunother. **37:**105–111.
48. **Wallis, R. S., M. Amir-Tahmasseb, and J. J. Ellner.** 1990. Induction of interleukin 1 and tumor necrosis factor by mycobacterial proteins: the monocyte Western blot. Proc. Natl. Acad. Sci. USA **87:**3348–3352.
49. **Wang, M. H., Y. Q. Chen, J. Gercken, M. Ernst, A. Bohle, H. D. Flad, and A. J. Vlmer.** 1993. Specific activation of human peripheral blood gamma/delta$^+$ T lymphocytes by sonicated antigens of Mycobacterium tuberculosis: role *in vitro* in killing human bladder carcinoma cell lines. Scand. J. Immunol. **38:**239–246.
50. **Wang, S., and T. Hazelrigg.** 1994. Implications for bcd mRNA localization from spatial distribution of exu protein in *Drosophila* oogenesis. Nature (London) **369:**400–403.
51. **Weller, M., K. Frei, P. Groscurth, P. H. Krammer, Y. Yonekawa, and A. Fontana.** 1994. Anti-Fas/APO-1 antibody-mediated apoptosis of cultured human glioma cells. Induction and modulation of sensitivity by cytokines. J. Clin. Invest. **94:**954–964.
52. **Wigley, C. B., and I. C. Summerhayes.** 1979. Loss of LETS protein is not a marker for salivary gland or bladder epithelial cell transformation. Exp. Cell Res. **118:**394–398.
53. **Wolf, S. A., D. E. Tracey, and C. S. Henney.** 1976. Induction of "natural killer" cells by BCG. Nature (London) **262:**584–586.