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Comparison of Immune Responses of Mice Immunized with Five Different *Mycobacterium bovis* BCG Vaccine Strains

MICHELINE R. R. LAGRANDERIE,[1] ANNE-MARIE BALAZUC,[1] EDITH DERIAUD,[2] CLAUDE D. LECLERC,[2] AND MARINA GHEORGHIU[1]*

Laboratoire du BCG[1] and Unité de Biologie des Régulations Immunitaires,[2] Institut Pasteur, 75724 Paris Cedex 15, France

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Among the various parameters which may contribute to *Mycobacterium bovis* BCG vaccination efficiency, the choice of the vaccine strain may play an important role. In the present study, we therefore compared the immunogenicity of five different BCG strains that are commonly used for BCG vaccine production (Glaxo 1077, Japanese 172, Pasteur 1173P2, Prague, and Russian strains). The comparison of the growth capacity of these BCG strains in BALB/c and C3H mice demonstrated that a great difference exists between the capacity of various BCG strains to multiply and persist in target organs. A much lower recovery of BCG could be shown in mice immunized with Prague and Japanese BCG strains. T-cell responses of BCG-immunized mice were also examined by analyzing T-cell proliferative responses, cytokine production, delayed-type hypersensitivity responses, and cytotoxic activity. All these assays demonstrated that BCG immunization induced strong $CD4^+$ T-cell responses, mostly of the Th1 type, as demonstrated by interleukin-2 and gamma interferon production. These studies also demonstrated that there are differences between BCG strains in stimulating these T-cell responses. A lack of induction of cytotoxic activity was observed following immunization with the Japanese strain. Lower anti-purified protein derivative antibody responses were also observed after intravenous or oral immunization with this BCG strain. Finally, the protective activity of these BCG strains was tested by measuring the capacity of immunized mice to eliminate recombinant Pasteur and Japanese BCG strains which expressed β-galactosidase. The results of these experiments clearly demonstrated that the Prague and Japanese strains were unable to protect mice against a second mycobacterial challenge whereas mice immunized with the Glaxo, Pasteur, or Russian strain eliminated the recombinant BCG very efficiently. Altogether, the results of the present study strongly support the view that there are considerable differences in the immunogenicity of various BCG vaccine strains and that these differences may play a major role in BCG vaccination efficiency.

Mycobacterium bovis BCG, since its discovery and first application as a vaccine to prevent tuberculosis in 1921 (4), has been subject to controversies. Different clinical trials estimated its protection efficiency to be from 0 to 80% (38). However, analysis of these divergent results (6, 26) demonstrated that methodological bias contributed to the conflicting data. Moreover, factors interfering with immune responses, such as contamination with environmental mycobacteria, genetic diversity of vaccinated populations, follow-up of tuberculosis cases in small children, and quality of BCG vaccine strains, may also interfere with the efficacy of BCG vaccination (3). However, in a recent meta-analysis of the published literature on the efficacy of BCG vaccine in the prevention of tuberculosis, it was found not only that the BCG vaccine prevented 80% of severe forms of tuberculosis but also that it gave 50% protection across many populations, forms of tuberculosis, and study designs (7).

It appears more difficult to estimate, from the results available from the few clinical comparative trials performed so far, the impact on protection of the origin of the BCG strain used for vaccination. One major problem is linked to the observation that protection varied among vaccines prepared with the same BCG vaccine strain (9). However, clinical observations of newborns demonstrated that a difference of reactogenicity exists among BCG strains (13). Following these observations, different independent (10, 17, 28) or interlaboratory experimental studies, undertaken under the aegis of the World Health Organization and the International Association for Biological Standardization, demonstrated the existence of differences between BCG strains in growth, colony morphology, residual virulence, and protection in animal models (32). These differences were attributed not only to BCG strain diversity but also to factors such as laboratory testing, BCG cultivation, and manufacture (1, 17, 32).

Various studies have shown that the Japanese 172, Brazilian, and Russian BCG strains have two copies of the insertion sequence IS*986*, secrete a 23-kDa protein, and contain methoxymycolate. In contrast, strains such as Pasteur 1173P2 and Danish 1331 carry a single copy of IS*986*, do not secrete the 23-kDa protein, and do not contain methoxymycolate (2, 12, 23). These results show that biochemical differences exist between BCG strains, which can be divided into at least two groups on the basis of these observations. However, it still remains to be determined if these various BCG vaccine strains have similar protective activity against tuberculosis. Differences in their immunogenicity may also be of major importance for the development of efficient recombinant BCG strains expressing foreign genes (33, 34, 37).

In the present study, to address these questions, we compared the cellular and humoral immune responses induced in mice by five BCG vaccine strains. We selected three strains which are the most commonly used in BCG vaccine produc-

* Corresponding author. Mailing address: Laboratoire du BCG, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France. Phone: (33.1) 45.68.82.35.

tion: Pasteur 1173P2, Japanese 172, and Glaxo 1077. We also included the Russian strain, which, like the Japanese strain, possesses two copies of the insertion sequence IS*986*, and the Prague strain, which was shown to have low potency against experimental tuberculosis in guinea pig (32).

MATERIALS AND METHODS

Animals. Specific-pathogen-free BALB/c and C3H mice (8 weeks old) obtained from Iffa-Credo, Saint-Germain sur l'Arbresle, France, were used in this study.

Microorganisms. The *M. bovis* BCG strains used in this study were Pasteur 1173P2, cloned in 1962 from a single colony (15); Glaxo 1077, derived from an ampoule of Danish BCG vaccine (strain passage 1077) in the 1950s; and Japanese 172 and the Russian and Prague strains, which were derived from the original Pasteur uncloned BCG culture strain. All these strains were sent to us freeze-dried by the World Health Organization as seed lots (30) and stored at −30°C.

The recombinant BCG (rBCG) strains expressing *lacZ* harbor pAM320 (25). This plasmid was transferred to *M. bovis* BCG Pasteur 1173P2 (25) and to *M. bovis* BCG Japanese 172 by electroporation (18a).

All these strains were grown as dispersed cultures in Beck-Proskauer medium supplemented with 6% glucose and 0.05% Triton 1331. Vaccine suspensions were prepared as previously described (18), and fresh-frozen vaccines were stored at −30°C until use. The number of CFU per milliliter was determined by plating suitable dilutions on Middlebrook 7H10 agar supplemented or not with kanamycin (10 μg/ml) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (1 μg/ml).

To avoid manufacturing and immunizing bias, each of the vaccine strains was prepared from seed lots in accordance with World Health Organization requirements (39). Strains were cultivated and manufactured in our laboratory by the same widely used standardized procedure, and for each BCG vaccine strain, an identical number of CFU was used for mouse immunization (14).

BCG growth in target organs. The growth of BCG strains was monitored by counting BCG and rBCG CFU in target organs of immunized mice as previously described (16). At various intervals after immunization, the spleens, Peyer's patches (PP), and lymph nodes of mice were homogenized (Stomacher LabBlender 80; Bioblock). Suitable dilutions were plated on Middlebrook 7H11 medium with or without kanamycin (10 μg/ml) or X-Gal (1 μg/ml) to determine the number of CFU of BCG or rBCG (*lacZ*), respectively. The number of rBCG (*lacZ*) CFU was determined by counting the blue colonies on X-Gal plates.

Analysis of cellular immune responses. At 14 days after subcutaneous immunization at the base of the tail with 10^9 CFU of each BCG strain, the draining inguinal lymph nodes were removed and a single-cell suspension was prepared and cultured in RPMI 1640 (Seromed, Berlin, Germany) supplemented with 2 mM L-glutamine, 50 μg of gentamicin per ml, 5×10^{-5} M 2-mercaptoethanol, and 10% fetal calf serum (Boehringer, Mannheim, Germany).

Proliferation assay. The proliferation assay was done as previously described (16). Briefly, cells were cultured at 8×10^5 cells per well in 96-well flat-bottom culture plates (Nunc, Roskilde, Denmark) in the presence of medium or of 10 or 100 μg of purified protein derivative (PPD; Ministery of Agriculture, Weybridge, England) per ml. Cells were incubated for 5 days at 37°C in humidified air containing 7% CO_2. During the last 20 h, 0.4 μCi of [^{3}H]thymidine per well (1 mCi/ml) (Amersham, Little Chalfont, United Kingdom) was added. The cells were harvested on fiberglass filters and washed with an Automash 2000 Dynatech washer (Bioblock), and then incorporated radioactivity was measured with a liquid scintillation counter (Beckman). The phenotype of proliferating cells was determined by adding to the cultures anti-CD4$^+$ (GK1-5) or anti-CD8$^+$ (H35-17-2) monoclonal antibodies (MAbs) as previously described (11, 24).

Assays for cytokine production. Mice were immunized as indicated in Results. Spleens were removed from normal or immunized mice, and single-cell suspensions were prepared in RPMI 1640 (Seromed, Munich, Germany) supplemented with 10% fetal calf serum, 2 mM glutamine (Seromed), 50 μM 2-mercaptoethanol, and antibiotics. A total of 4×10^6 cells were incubated with medium alone or either 2.5 μg of concanavalin A per ml or 10 μg of PPD per ml in a 1-ml volume in flat-bottom 24-well plates (Nunc, Roskilde, Denmark). Supernatants were harvested at 24 h for the interleukin-2 (IL-2) assay and at 72 h for the other cytokines. IL-2 was measured by using the CTLL cell line. Serial twofold dilutions of supernatants were added to CTLL cells in a 0.2-ml volume in 96-well plates. After 48 h, cultures were pulsed with [^{3}H]thymidine during the last 16 h of incubation and were harvested onto fiberglass filter paper. Radioactivity incorporation was measured in a liquid scintillation counter (Betamatic; LKB Wallac, Bromma, Sweden). The levels of IL-4, IL-5, IL-10, and gamma interferon (IFN-γ) were determined by sandwich enzyme-linked immunosorbent assay (ELISA) with BVD4-1D11, TRFK5, JES5-2A5, and R4-6A2 (Pharmingen, San Diego, Calif.), respectively, as capture antibodies. Then, 96-well microplates (Nunc) were coated with these MAbs by overnight incubation at 4°C. The plates were then blocked with 1% bovine serum albumin (BSA) (Boehringer) in phosphate-buffered saline (PBS) for 1 h at 37°C and were next incubated with each test supernatant for 1 h at 37°C. Then, the appropriate secondary biotinylated anti-cytokine-specific MAb (BVD6-24G2, TRFK4, SXC-1, or XMG2.2; Pharmingen) was added for 1 h at 37°C. The binding of the second MAb was detected with streptavidin-horseradish peroxidase (Amersham, Les Ulis, France). After three washes, the plates were developed by addition of *o*-phenylenediamine (Sigma) and hydrogen peroxide (Sigma). The reaction was stopped with sulfuric acid, and the plates were then read at 492 nm in an ELISA reader (Dynatech, Marnes la Coquette, France). All assays were standardized with recombinant murine cytokines. Recombinant IL-2 and IL-4 were obtained from Genzyme, Cambridge, Mass., whereas IL-5, IL-10, and IFN-γ were purchased from Pharmingen. Results were expressed in units per milliliter for IL-2 and in picograms per milliliter for the other cytokines.

Cytotoxicity assay. The cytotoxic activity of lymph node cells was tested as previously described (36) in a 4-h ^{51}Cr release assay. LN effector cells were harvested and cultured for 5 days in the presence of 10 μg of PPD per ml and 10 U of recombinant IL-2 per ml. The bronchoalveolar lavage (BAL) macrophages of the same mice were used as target cells. After 4 days of culture at 5×10^3 BAL macrophages per well in 96-well flat-bottom plates, 2 μCi of ^{51}Cr (Amersham) and 5×10^4 CFU of live BCG were added to each well. After overnight incubation, the target cells were washed and LN effector cells were added (100 μl per well) at different effector/target ratios. After 5 min of centrifugation at 1,000 rpm, the plates were incubated for 4 h at 37°C. A 100-μl volume of supernatant was then collected from each well, and the radioactivity was measured in counts per minute in a CliniGamma counter (LKB Pharmacia, Guyancourt, France). The percent specific lysis was calculated as follows: [(experimental radioactivity − spontaneous radioactivity)/(total radioactivity − spontaneous radioactivity)] × 100. The spontaneous radioactivity was determined with target cells incubated with RPMI 1640 alone, whereas the total radioactivity was determined with target cells incubated with 10% Triton X-100.

In some experiments, before the target cells were added, effector cells were incubated for 1 h at 37°C with medium or with anti-CD4 (GK1-5) or anti-CD8 (H-35-17-2) MAbs (20 μg/ml). Effector cells were then washed three times and incubated with target cells in the presence of GK1-5 or H-35-17-2 (1 μg/ml). The assay was then performed as previously described (11, 36).

Antibody assay. The immunoglobulin G (IgG), IgM, and IgA anti-PPD antibody responses were analyzed in BALB/c mice immunized either intravenously with two doses of 10^7 CFU at a 28-day interval or with 5×10^9 CFU administered orally. Sera and BAL and intestinal fluids were tested by ELISA as previously described (21). Briefly, microtiter trays (Nunc) were coated with 2 μg of PPD per well in PBS for 1 h at 37°C and 16 h at 4°C. After three washes, sera and fluids diluted in buffer (PBS, 0.1% Tween 20, 1% BSA) were added to the wells for 2 h at 37°C. After three washes, the wells were treated with goat anti-mouse IgG, IgM, or IgA alkaline phosphatase conjugates (Caltag Laboratories, San Francisco, Calif.). At 1 h later, 1 mg of *p*-nitrophenylphosphate per ml was added as substrate. After 30 min of incubation at 37°C, the plates were read photometrically at 405 nm in a micro-ELISA Autoreader (Dynatech, Bioblock). Titers were expressed as the $\log_{10}$ of the highest dilution that gave an A_{405} twice as high as that of nonimmunized mouse sera diluted 1/100. For BAL and intestinal fluids, titers were expressed as the $\log_{10}$ of the highest dilution that gave an A_{405} twice as high as that of nonimmunized mouse fluids diluted 1/10. The titers were calculated by using a computer-based logistic model with Microsoft Quick BASIC Software.

DTH. Delayed-type hypersensitivity (DTH) responses of BALB/c mice immunized intravenously (10^7 CFU twice), subcutaneously (10^9 CFU), or orally (5×10^9 CFU) were tested 1 and 3 months after immunization. Briefly, 50 μl of PPD (80 μg/ml) was injected into one footpad and 50 μl of PBS was injected into the contralateral footpad. The DTH reaction was quantified 48 h later by measuring the difference in millimeters between the footpad thicknesses of PPD- and PBS-injected footpads with a dial gauge caliper (sensitivity, 0.05 mm).

Statistics. The immune responses induced by the different BCG strains were compared by using the Mann-Witney U test; $P > 0.05$ was considered nonsignificant.

RESULTS

Comparison of the growth capacity of five different BCG strains. The capacity of BCG to multiply and persist in the target organs of the host is generally considered a prerequisite for inducing a good level of protective immunity against tuberculosis. We therefore first compared the growth capacity of five different BCG vaccine strains by enumeration of the CFU in the spleens of BALB/c mice immunized intravenously. As shown in Fig. 1, an increase in BCG CFU was observed 2 weeks after the injection of Pasteur, Russian, and Glaxo BCG strains. In contrast, no increase in CFU numbers was observed for the Japanese strain. An intermediate level of growth was observed for the Prague strain. At 2 weeks after injection, the difference between CFU recovered from spleens of mice given injections of Pasteur, Russian, and Glaxo strains was significant

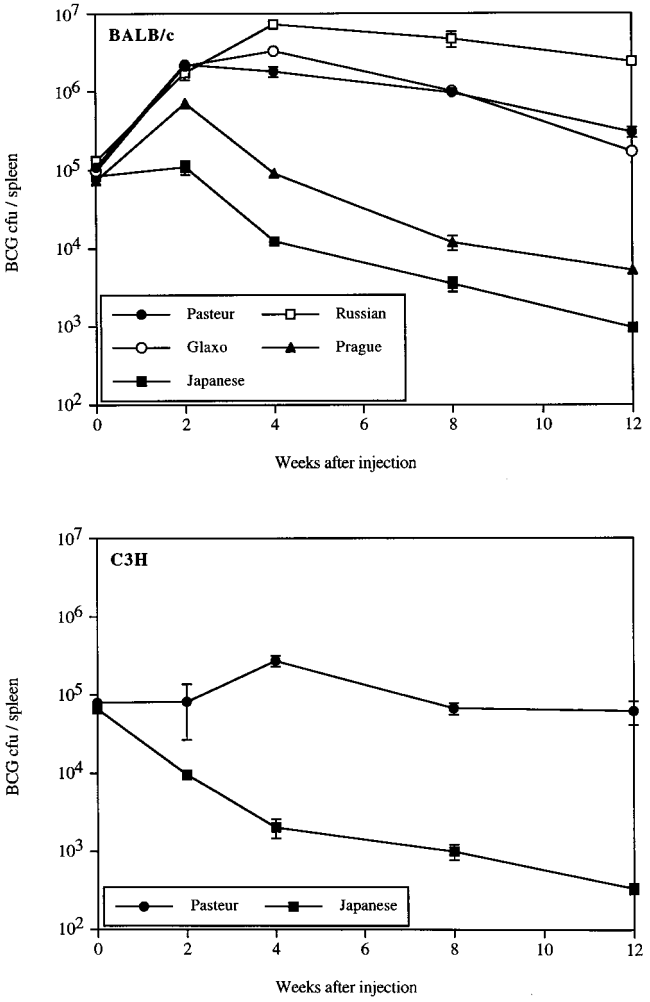

FIG. 1. BCG recovery in spleens of BALB/c and C3H mice immunized with different BCG vaccine strains. BALB/c or C3H mice were given intravenous injections of 10^6 CFU of different BCG vaccine strains. At various times after immunization, spleens of five mice per group were harvested and BCG recovery was determined at each time point. Results are expressed as mean CFU ± standard error. In both strains of mice, significant differences were found between the groups of mice immunized with the Pasteur, Russian, or Glaxo strain and the groups of mice immunized with the Prague or Japanese strain ($P \leq 0.05$).

compared with that for mice given injections of Japanese and Prague strains ($P < 0.05$). A significant difference was also observed between Japanese and Prague strains ($P < 0.05$).

One month after injection, the number of CFU recovered from the spleens of BALB/c mice immunized with Pasteur, Russian, and Glaxo strains remained unchanged. A very slow decrease of CFU numbers was observed for Pasteur and Glaxo strains at the end of the 3-month follow-up. The number of CFU recovered from spleens of mice immunized with the Russian BCG vaccine strain was still increasing 1 month after immunization and then persisted at a high level until the end of the experiment. In contrast, in mice injected with the Japanese and Prague strains, a decrease in the number of CFU was observed 1 month after immunization and the CFU numbers continued to decrease progressively to 10^3 during the following weeks.

The capacity of two of these strains, Japanese and Pasteur, to multiply and persist in the BCG-resistant C3H mouse strain was also analyzed (Fig. 1). Results similar to those found with BALB/c mice were obtained. The Japanese BCG strain did not multiply in C3H mice and was rapidly eliminated. A slight multiplication was observed for the Pasteur strain 1 month after injection, followed by stable CFU numbers until the end of the experiment.

We next analyzed the growth of these five BCG strains after oral immunization. As shown in Table 1, most of the ingested BCG (5×10^9 CFU) is eliminated in feces (10^7 CFU/g/24 h) and no significant difference was observed among the five BCG strains. During the hours following ingestion, a small number of CFU (10 to 300) could be recovered in Peyer's patches (PP) of mice vaccinated with the different BCG vaccine strains. The results suggest that BCG translocated from the gut through PP and reached the mesenteric lymph nodes (MLN) and then the spleen.

Persistence of BCG in the PP was observed during the weeks following injection only in mice immunized with the Pasteur or Russian strain. At 4 weeks after immunization, BCG could also be detected in the spleens of mice immunized with Pasteur, Glaxo, and Russian strains. It should be noted that 1 month after injection, BCG could be detected in the mesenteric lymph nodes only in mice immunized with the Pasteur strain.

Comparison of the protective capacity of five BCG strains against a challenge with recombinant BCG expressing β-galactosidase. To analyze the protective capacity of the five different BCG strains, we took advantage of Pasteur and Japanese rBCG strains which expressed the *lacZ* gene. The expression of β-galactosidase by these rBCG strains allowed us to discriminate between the immunizing BCG and the rBCG used for challenge (21, 25). In the first experiment, 4 months after immunization with the five different BCG strains, naive and immunized mice were challenged with Pasteur rBCG (*lacZ*). The resistance of mice to the growth of BCG was monitored by the enumeration of rBCG colonies in the mouse spleens at various times after injection. As shown in Fig. 2, a

TABLE 1. Recovery of BCG in target organs after oral immunization with various BCG vaccine strains

BCG strain	BCG recovery in target organs (CFU) at various times after immunization[a]							
	Feces (24 h)	PP			MLN		Spleen	
		6 h	2 wk	4 wk	2 wk	4 wk	2 wk	4 wk
Pasteur	$(7.6 \pm 0.5) \times 10^6$	292 ± 77	29 ± 16	0	25 ± 10	317 ± 184	8 ± 5	1,205 ± 305
Glaxo	$(8.2 \pm 0.7) \times 10^6$	36 ± 17	0	0	0	0	0	917 ± 544
Japanese	$(6.5 \pm 0.4) \times 10^6$	19 ± 6	0	0	0	0	0	0
Russian	$(6.6 \pm 0.5) \times 10^6$	27 ± 13	23 ± 12	0	37 ± 27	0	50 ± 25	990 ± 199
Prague	$(8.1 \pm 0.7) \times 10^6$	12 ± 10	0	0	0	0	8 ± 11	0

[a] BALB/c mice were orally immunized with 5×10^9 CFU of each BCG vaccine strain. The results are given as the mean number of CFU per mouse organ (MLN, mesenteric lymph nodes) and per gram of feces ± standard deviation ($n = 3$). BCG recovery in feces was analyzed in a separate experiment.

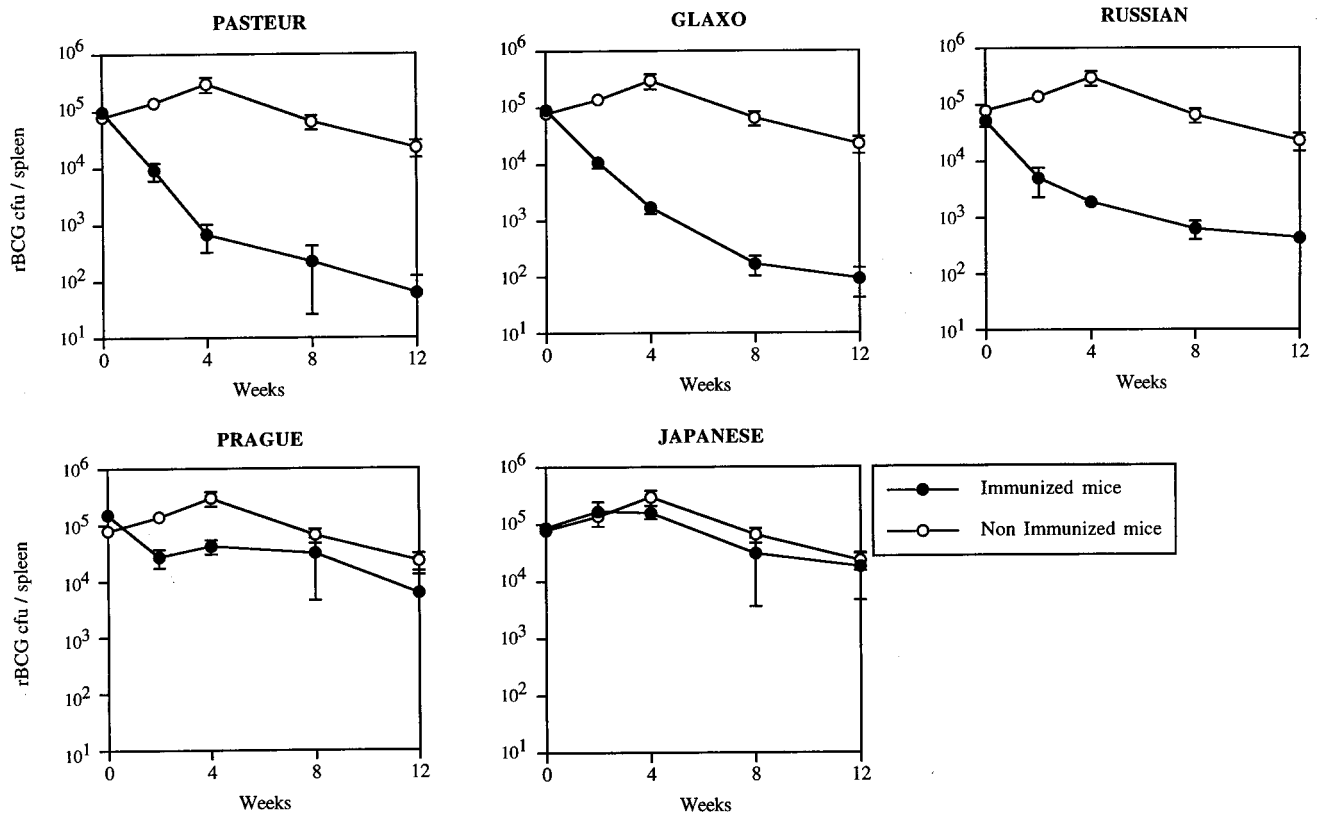

FIG. 2. Analysis of recovery of Pasteur rBCG (*lacZ*) in BALB/c mice immunized intravenously with different BCG vaccine strains. BALB/c mice were immunized intravenously with 10^6 CFU of various BCG vaccine strains. Control mice were left untreated. At 4 months later, all mice were challenged intravenously with 10^6 CFU of Pasteur rBCG (*lacZ*). At various times later, spleens of five mice per group were harvested and cultured in medium 7H11 supplemented with kanamycin and X-Gal. The number of rBCG colonies was determined by counting the blue colonies on X-Gal plates. Results are expressed as mean CFU ± standard error. A significantly higher rBCG elimination was observed in mice immunized with the Pasteur, Glaxo, or Russian BCG strain than in control groups ($P \leq 0.05$). No significant difference was observed in mice immunized with the Prague or Japanese strain and those in the control groups ($P > 0.05$).

strong growth inhibition of rBCG was observed for mice previously immunized with the Pasteur, Glaxo, and Russian strains compared with naive mice. Differences between rBCG growth in immunized and control mice were statistically significant ($P < 0.05$). In contrast, no inhibition of rBCG growth was observed in mice previously immunized with Prague and Japanese strains.

The low protective activity of the Japanese strain against the growth of Pasteur rBCG (*lacZ*) was confirmed in a second experiment, in which the immunogenicity of 10^6 or 10^7 CFU of either the Pasteur or the Japanese BCG strain was investigated in BALB/c and C3H mice. In this experiment, the number of recombinant BCG colonies recovered in the spleens of immunized mice was significantly larger after immunization with the Japanese strain than after immunization with the Pasteur strain. A protective efficacy of 91 to 99% was observed after injection of 10^6 or 10^7 CFU of the Pasteur strain into BALB/c or C3H mice, whereas in the same strains of mice, immunization with the Japanese strain gave a protective efficacy of only 13 to 42% (data not shown).

The low protective activity of the Japanese BCG strain was confirmed in a third experiment, in which we investigated the capacity of the Pasteur and Japanese strains to protect against a challenge with homologous or heterologous rBCG. As shown in Table 2, a much lower rBCG recovery was observed in control mice challenged with the Japanese rBCG than in those challenged with Pasteur rBCG. Immunization of BALB/c mice with Pasteur BCG induced an almost total protection against the growth of both Pasteur and Japanese rBCG strains. In contrast, a significantly lower protection (41 and 39%; $P < 0.05$) against the growth of these rBCG strains was observed in mice immunized with the Japanese BCG strain.

TABLE 2. Pasteur and Japanese BCG vaccine strains differ in their capacity to induce resistance to a challenge with homologous or heterologous recombinant BCG strains[a]

Immunization BCG strains	Challenge rBCG (*lacZ*) strain	rBCG recovery per spleen	
		CFU	% Protective efficacy[b]
None	Pasteur	68,333 ± 6,836	
Pasteur	Pasteur	7,170 ± 312	89
Japanese	Pasteur	39,830 ± 6,398	41[c]
None	Japanese	892 ± 82	
Pasteur	Japanese	0	100
Japanese	Japanese	542 ± 119	39[c]

[a] BALB/c mice (five per group) were immunized intravenously with 10^6 CFU of the Pasteur or Japanese BCG vaccine strain or were left untreated. At 4 months later, they were challenged intravenously with 10^6 CFU of Pasteur or Japanese rBCG (*lacZ*). Results represent the rBCG CFU ± standard error recovered in the spleens 2 months after challenge.

[b] Protective efficacy is calculated as follows: [(CFU in control mice − CFU in vaccinated mice)/CFU in control mice] × 100.

[c] The difference between protective efficacy of Pasteur and Japanese BCG strains was significant ($P < 0.05$).

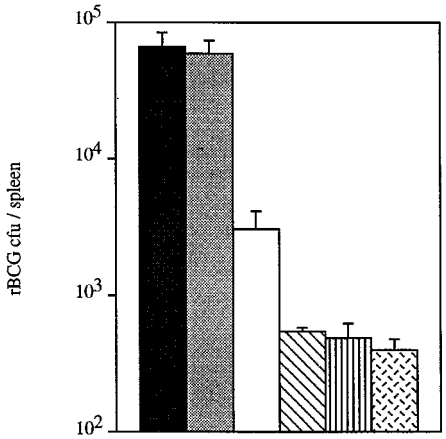

FIG. 3. Analysis of recovery of Pasteur rBCG (*lacZ*) in BALB/c mice immunized orally with different BCG vaccine strains. BALB/c mice were immunized orally with 5×10^9 CFU of various BCG vaccine strains. Control mice were left untreated. At 4 months later, all mice were challenged intravenously with 10^6 CFU of Pasteur rBCG (*lacZ*). At 3 months later, spleens of five mice per group were harvested and cultured in medium 7H11 supplemented with kanamycin and X-Gal. The number of rBCG colonies was determined by counting the blue colonies on X-Gal plates. Results are expressed as mean CFU ± standard error. A significantly higher rBCG elimination was observed in mice immunized with the Pasteur, Glaxo, Russian, or Prague BCG strain than in the control group ($P < 0.05$). No significant difference was observed in mice immunized with the Japanese strain and those in the control group ($P > 0.05$). Symbols: ■, control; ▨, Japanese; □, Prague; ▧, Russian; ▥, Glaxo; ▨, Pasteur.

The capacity of the five BCG vaccine strains to confer resistance to a challenge with the Pasteur rBCG (*lacZ*) strain in orally immunized mice was also analyzed, as shown in Fig. 3. Better protection was obtained with the Pasteur, Russian, and Glaxo strains than with the Prague strain, whereas the Japanese strain was totally ineffective.

Analysis of T-cell responses of mice immunized with five different BCG strains. (i) Proliferative responses and cytokine production. We next compared the T-cell responses of BALB/c mice immunized subcutaneously with 10^9 CFU of various BCG vaccine strains. We had previously demonstrated that LN cells of mice immunized under these conditions proliferated strongly in response to PPD and to antigens expressed by rBCG (21, 36, 37). Under these experimental conditions, high cytotoxic T-lymphocyte responses were also demonstrated (36). As shown in Table 3, a significant proliferative response was observed when LN cells of BCG-immunized mice were stimulated in vitro with PPD, whatever the BCG strain used for in vivo immunization. The PPD-specific proliferation was mostly due to the activation of $CD4^+$ T cells, as demonstrated by the strong inhibition (79 to 88%) of the proliferative response observed in the presence of anti-CD4 MAb.

After oral immunization, a low but significant proliferative response of mesenteric lymph nodes stimulated with PPD was also observed but only in mice immunized with Pasteur and Russian BCG vaccine strains (data not shown).

Proliferative responses to PPD were associated with the production of Th1 cytokines, such as IFN-γ and IL-2 (Table 4). No major difference in the production of these cytokines was observed after PPD stimulation of LN cells from mice immunized with the five BCG strains. A lower IFN-γ production was, however, observed in mice immunized with the Japanese strain, whereas IL-2 production was lower with LN cells from mice immunized with the Pasteur strain. It should be noted that IL-4, IL-5, and IL-10 were not detectable in any of these culture supernatants (data not shown).

(ii) DTH responses. We then analyzed the DTH responses of BALB/c mice immunized intravenously, subcutaneously, or orally with the five BCG strains. At 1 or 3 months after immunization, the DTH responses were analyzed by injection of PPD or PBS into mouse footpads. The highest DTH responses were observed in mice immunized subcutaneously (Fig. 4B). By using this route of immunization, all groups of mice developed high and comparable DTH responses.

In contrast, after oral vaccination (Fig. 4C), very strong DTH responses were observed in mice immunized with the Prague strain but very weak responses were observed in mice vaccinated with the Japanese strain. Other BCG strains gave intermediate DTH responses.

Very low DTH responses were observed in mice immunized intravenously, whatever the BCG strain used (Fig. 4A).

(iii) Cytotoxic T-cell responses. After in vitro stimulation with PPD of LN cells from BCG-immunized mice, high levels of cytotoxic activity against BCG-infected BAL macrophages could be demonstrated (Fig. 5). Such cytotoxic activity was mediated by $CD4^+$ T cells, as shown by inhibition of target cell lysis following incubation of effector cells with anti-CD4 MAb (Fig. 5B).

The highest level of specific lysis was observed when mice were immunized with Pasteur and Russian strains, whereas no detectable cytotoxic activity could be detected after immunization with the Japanese strain.

Analysis of anti-PPD-specific antibody responses. IgM, IgG, and IgA anti-PPD specific antibody responses were first analyzed in mice intravenously immunized on days 0 and 28 with 2×10^7 CFU of different BCG vaccine strains. As shown in Fig. 6, IgM and IgG antibody responses could be detected 1

TABLE 3. In vitro proliferation in response to PPD of LN cells from BCG-immunized mice[a]

BCG strain	Proliferation (cpm) in presence of:			% Inhibition of proliferation in presence of:	
	Medium	PPD		PPD and anti-CD4 MAb	PPD and anti-CD8 MAb
		10 μg/ml	100 μg/ml		
Pasteur	1,829 ± 349	6,855 ± 292 (3.7)[b]	18,741 ± 1,374 (10.0)	79 ± 1[c]	32 ± 2
Glaxo	990 ± 237	12,347 ± 1,820 (12.5)	14,172 ± 4,747 (14.3)	87 ± 2	17 ± 4
Japanese	883 ± 100	12,069 ± 1,406 (13.7)	15,975 ± 916 (18.1)	88 ± 1	11 ± 1
Russian	1,213 ± 190	16,339 ± 1,248 (12.4)	18,263 ± 409 (15.0)	88 ± 2	15 ± 2

[a] BALB/c mice (six per group) were immunized subcutaneously with 10^9 CFU of various BCG vaccine strains. For technical reasons, the Prague strain was not tested in this experiment. At 14 days later, inguinal LN cells were harvested and LN cell cultures were stimulated in vitro for 5 days with medium or with PPD either alone or in the presence of anti-CD4 or anti-CD8 MAb.

[b] Stimulation index = counts per minute with antigen/counts per minute without antigen. Values are given in parentheses.

[c] Percent inhibition of proliferation in the presence of 100 μg of PPD per ml and of medium or anti-CD4 or anti-CD8 MAb (10 μg/ml).

TABLE 4. IFN-γ and IL-2 production by lymph node cells from BALB/c mice immunized with various BCG vaccine strains[a]

Cytokine	In vitro stimulation with:	Cytokine production[b] for BCG vaccine strain:				
		Pasteur	Glaxo	Japanese	Russian	Prague
IFN-γ	Medium	44	44	141	493	13
	ConA	10,806	11,023	9,724	10,265	9,847
	PPD	8,610	6,507	3,693	7,729	6,322
IL-2	Medium	0	0	0	0	0
	ConA	2.0	2.2	4.0	2.4	2.0
	PPD	0.28	0.58	0.46	0.64	0.54

[a] BALB/c mice (six per group) were immunized subcutaneously with 10^9 CFU of various BCG vaccine strains. At 14 days later, LN cells were stimulated in vitro with medium. Concanavalin A (ConA) (2.5 μg/ml), or PPD (10 μg/ml). Supernatants were harvested 24 h (IL-2) or 72 h (IFN-γ) later.

[b] Production of IFN-γ is expressed in picograms per milliliter; production of IL-2 is expressed in units per milliliter.

month after immunization and further increased after the booster injection. These responses then remained stable until the end of the experiment. High levels of anti-PPD-specific IgG antibodies were observed in mice immunized with Pasteur, Glaxo, Prague, and Russian BCG strains, whereas significantly lower responses were obtained in mice immunized with the Japanese strain ($P < 0.05$). Similar results were obtained for the IgA antibody responses, which, however, remained low, even after two BCG injections.

Antibody responses were also analyzed in sera, intestinal secretions, and BAL lavage fluid of orally immunized mice (Fig. 7). This route of immunization induced low levels of circulating IgG antibodies compared with those induced by intravenous injection. The highest IgG and IgA anti-PPD antibody responses were observed in sera of mice immunized with the Pasteur and Russian strains. Low but detectable levels of anti-PPD-specific antibodies could also be demonstrated in intestinal secretions and BAL lavage fluid of mice immunized with the various BCG strains. At 16 weeks after oral immunization, these responses remained stable in mice immunized with the Pasteur BCG strain.

DISCUSSION

Different BCG strains are currently used to prepare vaccines against tuberculosis. Although some clinical observations and case-control studies suggested that differences in the protective activities of these various vaccine strains could exist, definitive evidence for such variation is still lacking (9, 35). It is presently almost impossible to compare the efficacy of different BCG vaccine strains in prospective human trials (3, 35).

In the present study, we therefore addressed this question by comparing the immunogenicity in mice of five different BCG strains, selected either because they are among the most commonly used in tuberculosis vaccination or because of their biochemical characteristics.

The first conclusion of this analysis is that these five BCG strains differ in their degree of multiplication and persistence in the host. Indeed, the Glaxo, Pasteur, and Russian strains were shown to multiply actively in mouse target organs and then to persist for months. In contrast, the Japanese and Prague strains were eliminated much more rapidly from the spleens of immunized animals. At 3 months after BCG injection, 10^3-fold more CFU were found in the spleens of mice immunized with the Russian strain than in spleens of mice immunized with the Japanese BCG strain. Such differences could play a major role in the induction and persistence of BCG-specific immune system responses. Although it is not established if such results obtained in mice could be extrapolated to human vaccination, it should be noted that the results were obtained in two different strains of mice which were previously described as being either sensitive (BALB/c mice) or resistant (C3H mice) to mycobacterial infection (31).

The behavior of these BCG strains was also monitored after oral administration in BALB/c mice. The results of this study demonstrated that most of the BCG was eliminated in the feces during the hours following oral administration and that residual BCG then reached the draining mesenteric lymph nodes and the spleen. Whereas Glaxo, Pasteur, and Russian strains were detectable in the spleens of orally immunized

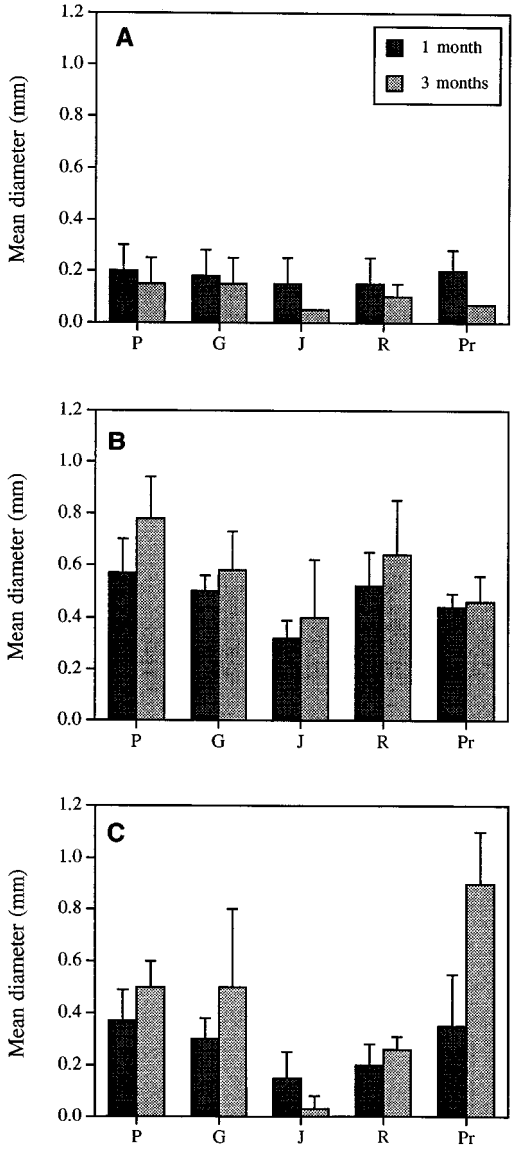

FIG. 4. PPD-specific DTH responses of BALB/c mice immunized with different BCG vaccine strains. BALB/c mice were immunized intravenously (10^7 CFU) (A), subcutaneously (10^9 CFU) (B), or orally (5×10^9 CFU) (C) with the Pasteur (P), Glaxo (G), Japanese (J), Russian (R), or Prague (Pr) vaccine strain. At 1 or 3 months after immunization, the DTH responses were analyzed. Results represent the mean increase in footpad thickness ± standard error for five mice per group.

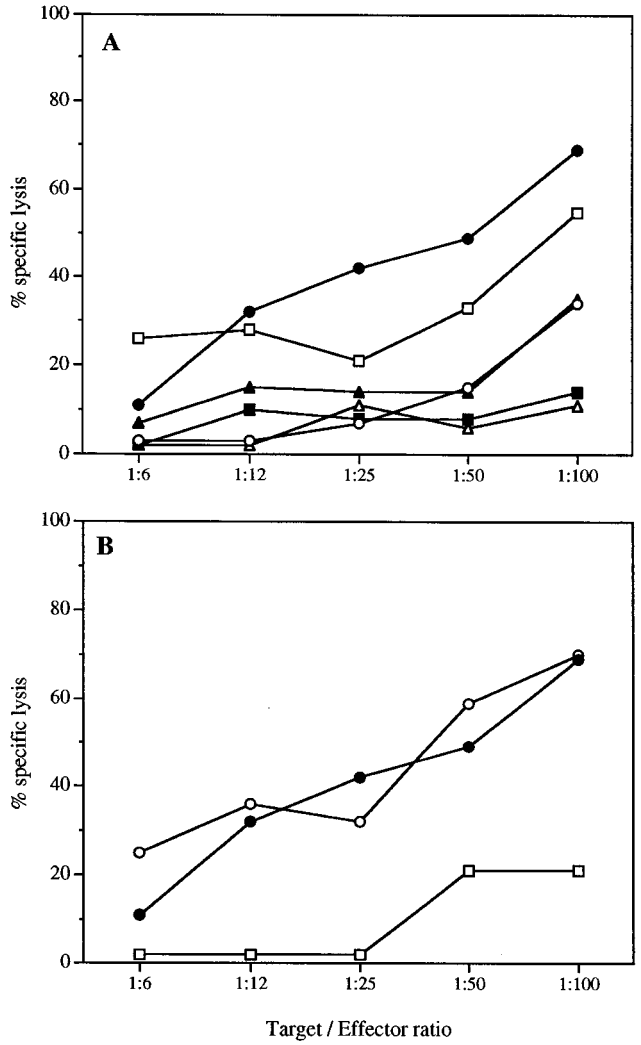

FIG. 5. BCG-specific cytotoxic activity of lymph node cells from BALB/c mice immunized with different BCG vaccine strains. BALB/c mice were subcutaneously immunized with 10^9 CFU of different BCG strains. At 2 weeks later, lymph nodes were removed and cells were cultured for 5 days with PPD. (A) The cytotoxic activity of these cultured cells was tested with, as target cells, bronchoalveolar macrophages harvested 2 weeks after immunization and infected with BCG. Noninfected macrophages incubated with effector cells obtained from Pasteur BCG-immunized mice were used as controls. Symbols: ●, Pasteur; ○, Glaxo; ■, Japanese; □, Russian; ▲, Prague; △, control. (B) The phenotype of cytotoxic cells was determined by adding medium or either anti-CD8 or anti-CD4 MAb to the effector cells. Symbols: ●, without MAb; □, anti-CD4 MAb; ○, anti-CD8 MAb. A significantly higher lysis was found with LN cells obtained from mice immunized with the Pasteur or Russian strain than with cells from mice immunized with the Prague BCG strain ($P \leq 0.05$). No lysis was found for LN cell cultures obtained from mice immunized with the Japanese strain vaccine.

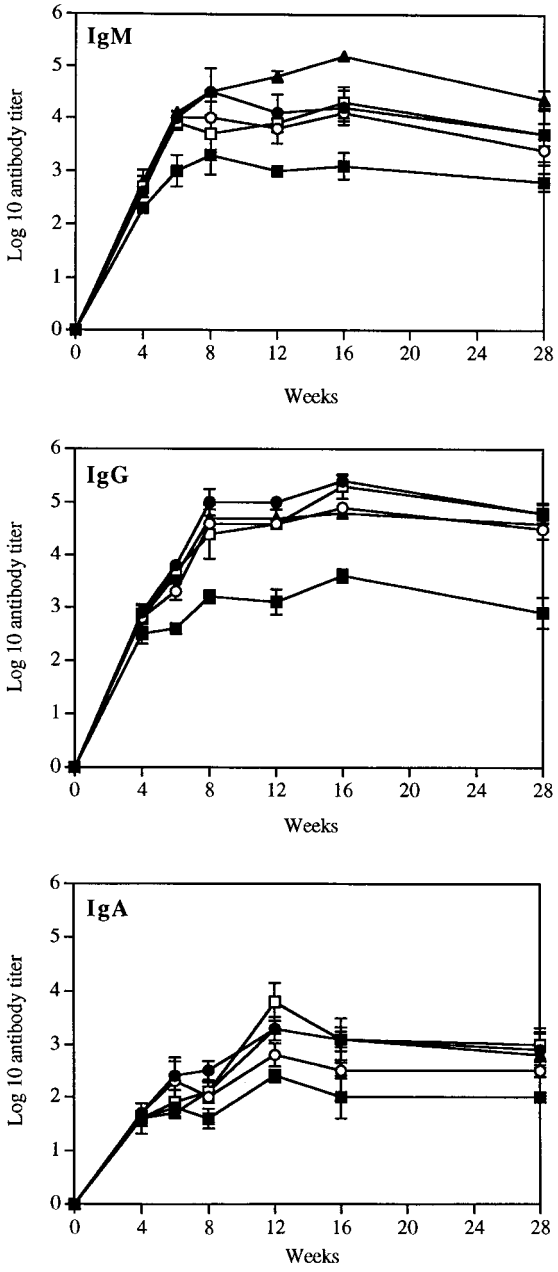

FIG. 6. Analysis of anti-PPD antibody responses of BALB/c mice immunized with different BCG vaccine strains. BALB/c mice were intravenously immunized on days 0 and 28 with 2×10^7 CFU of different BCG vaccine strains. At various times after immunization, mice were bled and IgM, IgG, and IgA anti-PPD antibody titers were determined by ELISA. Each point represents the mean $\log_{10}$ titer ± standard error for five serum samples per group. Symbols: ●, Pasteur; ○, Glaxo; ■, Japanese; □, Russian; ▲, Prague.

mice, no organisms could be detected in the spleens of mice which received the Japanese or Prague BCG strain. These results therefore confirmed that BCG strains could differ in their capacity to colonize and persist in the host, regardless of the route of administration.

These results were strongly correlated with the protective activity of these various BCG strains, expressed in terms of their capacity to confer resistance to the growth of recombinant Pasteur and Japanese BCG strains expressing β-galactosidase. Whereas the Pasteur rBCG strain was actively eliminated in mice immunized with the Glaxo, Pasteur, or Russian strain, its growth was not significantly modified in mice immunized with the Prague or Japanese BCG strain. Identical results were found when mice immunized with the Pasteur or Japanese BCG strain were challenged with either Pasteur or Japanese rBCG.

These findings support the view that the induction of protective immune responses against mycobacteria requires the multiplication and long-term survival of the vaccinating organisms in the host (8, 22, 27). We therefore analyzed the immune responses induced by the five different BCG strains to try to establish a correlation between some of these immune re-

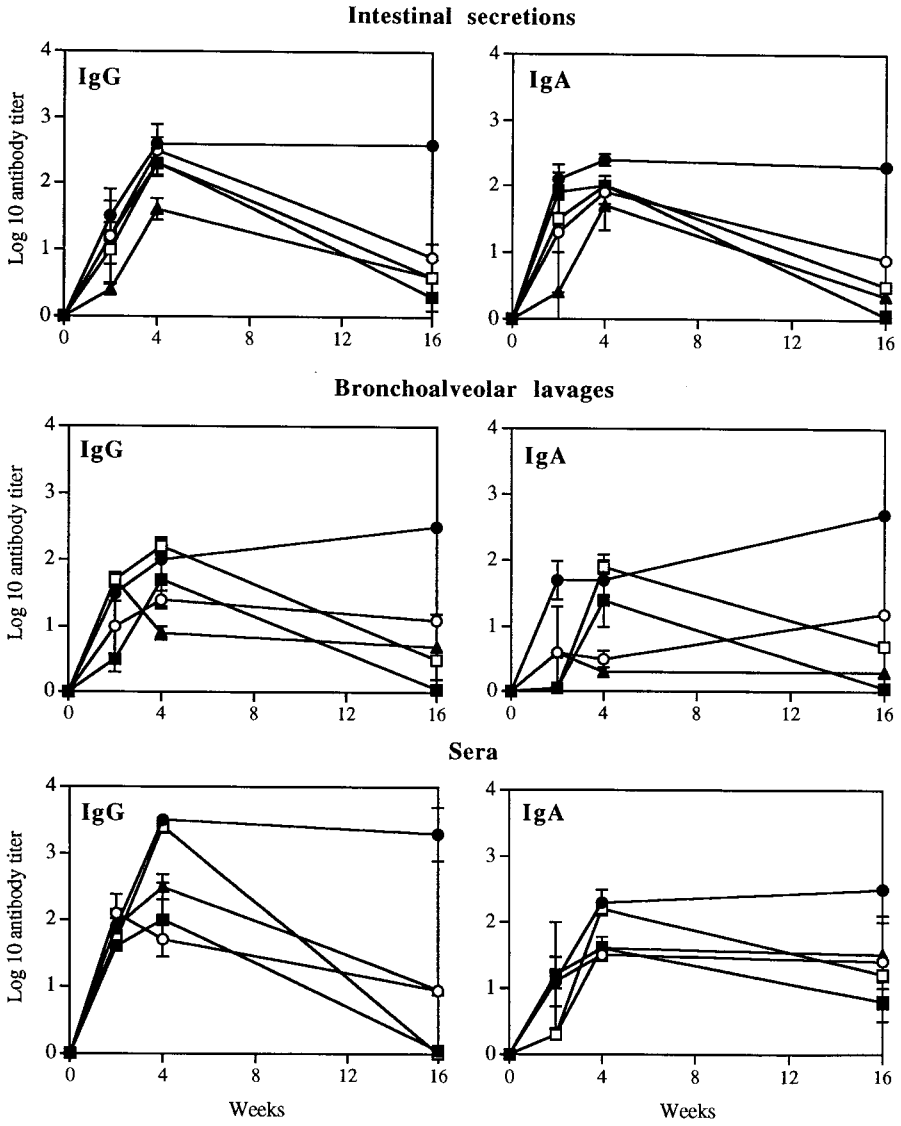

FIG. 7. Analysis of anti-PPD antibody responses in sera and intestinal and BAL fluids of BALB/c mice immunized orally with different BCG vaccine strains. BALB/c mice were immunized orally with 5×10^9 CFU of different BCG strains. At various times after immunization, IgG and IgA anti-PPD antibody responses of sera and intestinal and BAL fluids were determined by ELISA. Results represent the mean $\log_{10}$ titer ± standard error for five samples (sera or fluids) per group. Symbols: ●, Pasteur; ○, Glaxo; ■, Japanese; □, Russian; ▲, Prague.

sponses and the protective activity induced by BCG immunization.

No major differences were found between the capacities of the five BCG strains to induce proliferative responses against PPD. The analysis of cytokine production after PPD stimulation of BCG-immunized mouse LN cells also failed to demonstrate the existence of major differences in the T-cell responses induced by the different BCG strains. In contrast to these results, the analysis of cytotoxic activity demonstrated that the Pasteur and Russian strains induced stronger cytotoxic T-cell responses than did the Prague and Glaxo strains. Immunization of mice with the Japanese BCG strain was totally ineffective in inducing cytotoxic T-cell responses. The protective Glaxo strain did not induce higher cytotoxic T-cell responses than the nonprotective Prague strain. These results therefore support the hypothesis that the observed inhibition of rBCG growth was mediated mostly by the intracellular killing or growth inhibition of these microorganisms by activated macrophages, and the exact role of BCG-primed cytotoxic T cells remains to be determined (5, 19, 29). It should, however, be noted that the immunization conditions required to analyze these various immune responses are different. It is therefore difficult to establish a definite correlation between the cellular immune responses measured and the levels of protective immunity induced by the various BCG vaccine strains.

The capacities of the five different BCG vaccine strains to induce antibody responses were also shown to be significantly different. After intravenous immunization, the Japanese BCG strain induced only very low levels of anti-PPD-specific antibodies. However, no such difference was observed for the Prague BCG strain, which, like the Japanese strain, is unable to stimulate growth inhibition of rBCG. Therefore, there does not seem to be a correlation between the induction of antibody

responses by BCG immunization and the stimulation of protective immunity.

Indeed, it is generally considered that antibodies play a minor role in the immune protection against tuberculosis. However, antibodies may facilitate mycobacterial elimination by increasing opsonization of the microorganisms (20).

Finally, this study demonstrated that the immunogenicity of these BCG strains is not related to biochemical differences such as lipid content or IS*986* copy number. These five BCG strains, prepared under similar experimental conditions, clearly differ in their capacity to survive in the host organism and to trigger protective immune responses. These differences seem to be related to the genetic characteristics of the BCG strains rather than to the route of administration or to genetic differences of the host.

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