A major goal of current vaccine development is the induction of strong immune responses against protective antigens delivered by mucosal routes. One of the most promising approaches in that respect relies on the use of live recombinant vaccine carriers. In this study, Mycobacterium bovis BCG was engineered to produce an intracellular glutathione S-transferase from Schistosoma haematobium (Sh28GST). The gene encoding Sh28GST was placed under the control of the mycobacterial hsp60 promoter on a replicative shuttle plasmid containing a mercury resistance operon as the only selectable marker. The recombinant Sh28GST produced in BCG bound glutathione and expressed enzymatic activity, indicating that its active site was properly folded. Both intraperitoneal and intranasal immunizations of BALB/c mice with the recombinant BCG resulted in strong anti-Sh28GST antibody responses, which were enhanced by a boost. Mice immunized intranasally produced a mixed response with the production of Sh28GST-specific immunoglobulin G1 (IgG1), IgG2a, IgG2b, and IgA in the serum. In addition, high levels of anti-Sh28GST IgA were also found in the bronchoalveolar lavage fluids, demonstrating that intranasal delivery of the recombinant BCG was able to induce long-lasting secretory and systemic immune responses to antigens expressed intracellularly. Surprisingly, intranasal immunization with the BCG producing the Sh28GST induced a much stronger specific humoral response than intranasal immunization with BCG producing the glutathione S-transferase from Schistosoma mansoni, although the two antigens have over 90% identity. This difference was not observed after intraperitoneal administration.

Mucosal vaccination consists in delivering antigens at mucosal sites for the induction of mucosal and/or systemic immune responses. A major advantage of this approach over traditional parenteral immunizations is its noninvasiveness, even for the induction of systemic immune responses. Noninvasive vaccination strategies are associated with fewer side effects, which significantly increases user compliance. However, one of the important limitations of mucosally applied antigens is their poor immunogenicity (6). The use of recombinant live vectors able to colonize mucosal surfaces and to deliver foreign antigens to the mucosa-associated lymphoid tissues is an efficient tool to overcome this hurdle. Consequently, several live vaccine delivery systems have been developed in recent years (for a review, see reference 11).

Most efforts have so far been devoted to the development of vectors that can be administered orally. However, this route of immunization requires usually high doses and repeated administrations, due to the instability of the antigens and the vectors in the proteolytic and acidic environment of the stomach, and also due to strong ecological competition of live vectors with resident commensal microorganisms in the gastrointestinal tract. An attractive alternative to the oral route is the intranasal (i.n.) route of immunization. The respiratory tract is a less acidic and proteolytic environment and is less colonized by microorganisms than the gastrointestinal tract. Furthermore, it has been documented that on a dose basis, i.n. administration is more effective than oral administration (10).

One of the most widely used live vaccines is the bacille de Calmette et Guérin (BCG), an attenuated Mycobacterium bovis strain used for human vaccination against tuberculosis (5). Although this vaccine is generally given subcutaneously, it has also been widely administered orally. However, since M. bovis is essentially a respiratory pathogen, BCG may be better adapted for i.n. administration.

More recently, BCG has also been used for the delivery of foreign antigens (12). Recombinant BCG strains were able to induce both humoral and cellular immune responses against the foreign antigens in various experimental models (15, 19, 27, 32). In most studies, the recombinant microorganisms were given parenterally. In this work, we describe the humoral antibody responses elicited after i.n. administration of recombinant BCG producing the 28-kDa glutathione S-transferase from Schistosoma haematobium (Sh28GST) as a model antigen. Infection in human populations with S. haematobium, the etiologic agent of urinary schistosomiasis, results in severe and frequently irreversible damage to urinary tract (9). The pathology of schistosomiasis is mainly caused by a massive egg output, and protection in humans has been correlated with the presence of antibodies capable of neutralizing the enzyme ac-
tivity of the schistosomal GSTs (23). Here, we show that i.n. vaccination of mice with the recombinant BCG can elicit high levels of neutralizing anti-Sh28GST serum antibodies, as well as high levels of specific mucosal immunoglobulin A (IgA) in bronchoalveolar lavage fluids (BALF). Interestingly, this anti-Sh28GST immune response was much stronger than that induced against the Schistosoma mansoni 28-kDa GST (Sm28GST) elicited by i.n. administration of recombinant BCG producing Sm28GST, although the proteins are approximately 90% identical in amino acid sequence (28).

**MATERIALS AND METHODS**

**Plasmids and DNA manipulation.** Plasmids pUC::Jx660/ KpnI, and pEN005 (15) were described previously. M13H9, a M13mp18-derivative containing the Sh28GST CDS (28), was generously provided by F. Trottein (Institut Pasteur de Lille, Lille, France), and pUC18 was purchased from New England Biolabs (Beverly, Mass.). Analyses of plasmids from mycobacteria were done by electrophoresis in E. coli DH5α, E. coli Bl21, E. coli LG04, La Jolla, Calif. Expression of Sh28GST, we used the M. bovis vaccinia2 strain 1173P2 (World Health Organization, Stockholm, Sweden). BCG was transformed as previously described (13), and recombinant BCG(pEN005) were selected by their resistance to HgCl₂.

**HindIII and was then inserted into the pEN103 shuttle vector previously digested with the binding site, and ATG initiating codon, as well as the Sh28GST-coding sequence.**

**PCR from M13H9, using primers 5'-AATTCGAGCTCGTGCATACTACAGGTATAC-3' and 5'-GAATATCCAGCAGCTGTGATCATATC**

**Restriction enzymes and T4 DNA ligase were purchased from Boehringer (Mannheim, Germany). All DNA manipulations were performed by using standard protocols as described by Sambrook et al. (25).**

**Bacterial strains and growth conditions.** All cloning steps were performed in E. coli DH5α, E. coli Bl21, E. coli LG04, La Jolla, Calif. Expression of Sh28GST, we used the M. bovis vaccinia2 strain 1173P2 (World Health Organization, Stockholm, Sweden). BCG was transformed as previously described (13), and recombinant BCG(pEN005) were selected by their resistance to HgCl₂, and ATG initiating codon and cloned into HindIII KpnI restriction site (underlined), and the second primer contains a KpnI restriction site (underlined). The PCR fragment was then digested with NcoI and KpnI and cloned into NcoI KpnI-restricted pUC::Jx660, giving rise to pUC::Jx660/Sh28GST.

**Construction of the Sh28GST expression vector.** To construct pUC::Jx660/ Sh28GST, a 666-bp fragment containing the Sh28GST gene was amplified by PCR from M13H9, using primers 5'-AATTCGAGCTCGTGCATACTACAGGTATAC-3' and 5'-GAATATCCAGCAGCTGTGATCATATC**

**To evaluate specific IgA responses in BALF, ELISAs were performed as described above except that after coating, the plates were washed once with PBS containing 0.05% Tween 20 and saturated with 5% nonfat dry milk in PBS.** Titers were determined by ELISA as previously described (15), using 20 μg of Sh28GST or Sm28GST per ml or 25 μg of soluble BCG antigens per ml for coating, and 50 μl of peroxidase-conjugated goat anti-mouse IgG, IgG1, IgG2a, and IgG2b (Southern Biotechnology, Birmingham, Ala.) at 1/17,000, 1/10,000, 1/12,000, and 1/12,000 dilutions, respectively, as secondary antibodies. For IgG determination, ELISAs were performed as described elsewhere (21). Titers were defined as the highest dilution yielding an absorbency three times above the background.

**RESULTS**

**Expression of Sh28GST in BCG.** For the production of Sh28GST in BCG, we used pENSh28, a plasmid containing the Sh28GST-encoding gene under the control of the BCG hsp60 promoter and containing mercury resistance genes (2, 4) as the only selectable marker to avoid potential dissemination of antibiotic resistance genes to other bacteria. The expression level of Sh28GST in BCG(pENSh28) was analyzed and compared to that of Sm28GST in BCG(pEN005) (15) by immunoblotting of whole-cell lysates using a rat antisemir raised against peptide 190-211 of Sh28GST. This epitope is highly conserved between Sm28GST and Sh28GST and contains only two conservative substitutions: Asp by Asn in position 205 and Ala by Pro in position 207 (28). Therefore, this antipeptide antisemir is highly cross-reactive. As shown in Fig. 1A, a protein reactive with this antisemir and of the expected size was readily detected in recombinant BCG producing the Sh28GST, as well as in BCG producing Sm28GST. In addition, the two strains produced similar amounts of the recombinant antigen. As expected, no immune-reactive band was detected in untransformed BCG.

**Purification and enzymatic activity of Sh28GST expressed in E. coli.** Xu et al. (31) have shown that protective immunity against S. mansoni is associated with antibodies blocking the active site of Sm28GST, suggesting that the conservation of the three-dimensional structure of the active site is important for the induction of a protective antibody response. We therefore tested the functional integrity of the Sh28GST active site by...
binding of the protein to GSH and by the expression of its activity. Similar to previous studies on BCG-produced Sm28GST (15), total-cell extracts of BCG(pENSh28) were subjected to affinity chromatography using a GSH-agarose column. Eluted fractions were pooled and analyzed by SDS-PAGE. Figure 1B shows that Sh28GST produced by recombinant BCG and purified in a single step by GSH-agarose chromatography comigrated exactly with the protein purified from yeast used as a positive control. Assessment of the enzymatic activity of Sh28GST purified from BCG indicated that it was essentially indistinguishable from that expressed by Sh28GST purified from yeast (Fig. 1C). These results imply that Sh28GST produced in BCG has conserved the three-dimensional structure of its active site.

Antibody responses induced after i.p. immunization with recombinant BCG in mice. We previously showed that i.p. administration of BCG(pEN005) induced a strong antibody response against Sm28GST in BALB/c mice (15). To test whether recombinant BCG producing Sh28GST is also able to induce a specific response against Sh28GST, groups of four BALB/c mice were immunized i.p. with three different doses (10^8, 5 × 10^7, and 5 × 10^6 bacteria) of either untransformed BCG or BCG(pENSh28). Specific anti-BCG and anti-Sh28GST antibody responses were analyzed by ELISA. Figure 2A shows that the highest dose of recombinant or untransformed BCG induced the strongest anti-BCG antibody responses after a single immunization. Lower responses were obtained with 5 × 10^7 BCG, whereas no response was observed with 5 × 10^6 BCG. A second administration of 10^8 BCG to mice that had received the highest dose was accompanied by a slight increase in anti-BCG antibody levels, and this response remained very stable for at least 19 weeks after the boost. A second administration of 5 × 10^6 or 5 × 10^5 BCG to mice that had received smaller doses was accompanied by a much stronger increase of the antibody responses against BCG. These responses also remained stable for at least 19 weeks after the boost.

Recombinant BCG but not untransformed BCG was able to induce a significant anti-Sh28GST antibody response after a single immunization, regardless of the dose used (Fig. 2B). Interestingly, the intermediate dose (5 × 10^7 BCG) induced the strongest response. In all three groups of mice immunized with the recombinant BCG, the anti-Sh28GST responses were significantly enhanced after the second immunization.

To directly compare the anti-GST antibody levels induced by i.p. administration of BCG producing Sh28GST with those induced by i.p. administration of BCG producing Sm28GST, groups of 4 BALB/c mice were immunized i.p. with 5 × 10^8 BCG(pENSh28) or BCG(pEN005) and boosted in the same way 8 weeks later. Two weeks after the boost, serum antibodies were collected and analyzed. As shown in Fig. 3, i.p. immunization with either strain resulted in comparable titers of anti-GST antibodies.

Antibody responses induced after i.n. immunization with recombinant BCG in mice. To investigate whether BCG(pENSh28) was also able to induce a specific serum anti-Sh28GST antibody response after i.n. administration, BALB/c mice were immunized i.n. with approximately 10^7 recombinant or untransformed BCG and boosted with the same dose 16 weeks later. Results of ELISA showed important anti-BCG antibody responses already 4 weeks after a single immunization with either BCG strain. These responses were substantially higher 16 weeks after the immunization (Fig. 4A). The booster doses only slightly further increased the anti-BCG responses, which remained stable for at least 12 weeks after the second immunization.

Anti-Sh28GST antibodies were induced only after administration of BCG(pENSh28). The primary response induced af-
After a single i.n. immunization was significantly enhanced after the boost and remained stable for at least 12 weeks after boost. No specific Sh28GST antibodies were detected in mice immunized with untransformed BCG (Fig. 4B). Interestingly, the anti-Sh28GST titers obtained after i.n. administration of the recombinant BCG were as high as those obtained after i.p. immunization with the same strain, indicating that i.n. administration of recombinant BCG is an efficient way to induce high levels of serum antibodies against a foreign antigen.

Analyses of the isotypic profiles of the anti-Sh28GST antibodies after i.n. immunization with BCG(pENSh28) indicated a mixed response, characterized by low titers of IgG1 and higher titers of IgG2a and IgG2b after the first immunization (Fig. 5). The IgG1 response increased significantly after the second immunization. The boost was also followed by a significant increase in the IgG2b level which decreased progressively, whereas the IgG2a response remained stable. A low but constant level of serum anti-Sh28GST IgA titers was also detected after the booster dose.

IgA responses in BALF of i.n.-immunized mice. To investigate whether i.n. administration of recombinant BCG could also induce a specific IgA response in BALF, total IgA as well as anti-BCG and anti-Sh28GST antibodies were measured in BALF before and after i.n. administration of BCG(pENSh28). Figure 6A shows that BALF from nonimmunized mice contained small but detectable amounts of total IgA. A single nasal administration of either untransformed or recombinant BCG induced a marked increase in total IgA, which was not enhanced by a second immunization and remained constant for at least 12 weeks after the boost.

Specific anti-BCG IgA in BALF were detectable after a single nasal administration of either untransformed or recombinant BCG (Fig. 6B), and a second immunization was accompanied by an important increase of this response. A specific anti-Sh28GST IgA response in BALF was observed only in mice immunized with the recombinant BCG (Fig. 6C). This response was strongly increased by the booster dose and declined 12 weeks after the boost, although it was still detectable.

Sh28GST and Sm28GST cross-reactivity after i.n. immunization with recombinant BCG. To compare the anti-Sh28GST
antibody response induced by BCG(pENSh28) with the anti-Sm28GST antibody response induced by BCG(pEN005), mice were immunized i.n. with either strain, and the sera were analyzed by ELISA. Surprisingly, the mice immunized i.n. with BCG(pEN005) produced only low levels of anti-Sm28GST antibodies, whereas the mice immunized with BCG(pENSh28) produced 10- to 100-fold-higher serum anti-Sh28GST antibody titers (Fig. 7), although the proteins exhibit approximately 90% amino acid sequence identity. In addition, the sera from the mice immunized with BCG(pEN005) contained only low levels of Sh28GST-cross-reactive antibodies. In contrast, the sera from the mice immunized i.n. with BCG(pENSh28) contained very high levels of cross-reactive antibodies against Sm28GST. Anti-BCG antibody titers were comparable in all groups of animals, indicating that they all were exposed to a similar dose of BCG.

![Graphs showing antibody responses](image)

**FIG. 4.** Antibody responses induced after i.n. immunization. BALB/c mice were immunized i.n. with $10^7$ untransformed BCG or BCG producing Sh28GST (BCG/Sh28GST) and boosted 16 weeks later in the same way. The sera from each group were collected 4 (black bars) or 16 (gray bars) weeks after the first immunization or 6 (white bars) or 12 (hatched bars) weeks after the boost, pooled, and analyzed by ELISA using BCG total soluble proteins (A) or purified Sh28GST produced in yeast (B).

**FIG. 5.** Anti-Sh28GST antibody isotype profiles elicited after i.n. immunization with BCG producing Sh28GST. BALB/c mice were immunized i.n. with $10^7$ BCG organisms producing Sh28GST (BCG/Sh28GST) and boosted 16 weeks later in the same way or left unimmunized (control). The sera from each group were collected 4 (black bars) or 16 (gray bars) weeks after the first immunization or 6 (white bars) or 12 (hatched bars) weeks after the boost, pooled, and analyzed by ELISA for the presence of specific anti-Sh28GST IgG1, IgG2a, IgG2b, and IgA isotypes.
mice were immunized i.n. with $10^7$ untransformed BCG or BCG producing
specific responses were ruled out by the absence of swelling in comparable DTH responses 48 h after the challenge. Nonspecific immune responses were inhibited in a dose-dependent manner in the presence of sera from mice immunized with BCG(pENSh28), whereas no significant inhibition was observed with sera from noninfected mice after the challenge. These results suggest that the expression of Sh28GST in BCG does not significantly alter its capacity to induce a DTH response.

**DISCUSSION**

Most antigens are poorly immunogenic when delivered by the mucosal route, probably because they fail to interact efficiently with the mucosa-associated lymphoid tissues (6). However, mucosal immunization presents several advantages over parenteral immunization, including the induction of secretory antibodies and the ease of delivery. To increase immunogenicity of mucosally delivered antigens, live vectors such as the most widely studied *Salmonella* vector have been developed (for a recent review, see reference 11). The mucosal route most extensively explored so far is the oral route. It has recently been shown that recombinant BCG can be given orally to induce local and systemic cytotoxic T-lymphocyte responses in mice (18). However, efficient oral immunization requires several consecutive and high doses of live bacteria. BCG was originally given orally to vaccinate against tuberculosis. However, this route was discontinued because the viability of BCG is reduced by 1 to 2 logs by exposure to gastric secretions and low pH (7), and thus very high doses of BCG were required. In addition, oral administration of BCG was frequently associated with cervical lymphadenopathy. Intranasal administration may constitute an interesting alternative. Several studies have provided evidence that aerosol vaccination with BCG, at doses comparable to those used here (approximately $10^6$ BCG organisms/animal), was superior to immunization by other routes in the protection of primates against tuberculosis (1). In addition, aerosol-vaccinated animals showed no obvious adverse effects. No adverse effects were seen when BCG was given by aerosol to human volunteers, including young children, with up to $10^6$ organisms inhaled (24).

Despite these advantages, only a few studies have reported on the immune responses elicited against heterologous antigens after i.n. administration of recombinant BCG. Langermann et al. (20) have demonstrated that recombinant BCG producing OspA of *Borrelia burgdorferi* induces strong systemic and mucosal immune responses in BALB/c mice. However, the immunogenicity of this protein strongly depended on its surface exposure as a lipoprotein. Nonlipidated intracellularly expressed OspA was only poorly immunogenic when delivered by the nasal route.

In this study, we have constructed a recombinant BCG strain producing Sh28GST in a nonlipidated intracellular form. The gene encoding Sh28GST was placed downstream of the hsp60 promoter in a replicative shuttle plasmid containing mercury resistance determinants as the only selectable markers. The absence of antibiotic resistance markers in this vector avoids possible dissemination of antibiotic resistance, were such strains to be used in humans or animals. Sh28GST produced by this strain retained its GSH binding capacity and catalyzed the transferase reaction, strongly suggesting that it had folded properly in BCG.

Intranasal administration of this recombinant BCG strain induced strong and sustained systemic and mucosal immune responses against the antigen. The systemic immune response against Sh28GST was at the same level as that obtained after i.p. immunization with the recombinant BCG, a route traditionally considered as one of the most efficacious (15). These results indicate that i.n. administration of recombinant BCG can elicit strong immune responses against heterologous antigens even if they are not surface associated or lipidated. However, in contrast to i.p. immunization, i.n. delivery also induced...
a mucosal IgA response against Sh28GST. Naive BALB/c mice contain only small amounts of IgA in BALF. Intranasal immunization with BCG was followed by a drastic increase in the total mucosal IgA levels in BALF. BCG-specific and Sh28GST-specific IgA were also detected in BALF, even after a single immunization. This response was greatly enhanced after a second i.n. immunization and remained high for at least 12 weeks after the boost. The induction of anti-Sh28GST IgA is particularly interesting, since Ndhlovu et al. (22) have recently shown that IgA may play an active role in resistance to S. haematobium infection in humans. This is consistent with previous observations by Grzych et al. (8) on the potential role of anti-Sm28GST IgA in human schistosomiasis caused by S. mansoni.

In addition to the IgA response, i.n.-immunized mice also developed strong and long-lasting serum IgG responses. The anti-Sh28GST isotype profile showed the presence of IgG1, IgG2a, and IgG2b, suggestive of a mixed response. The booster immunization was accompanied by a progressive increase of the IgG1 isotype, whereas IgG2a levels remained stable for at least 12 weeks after boost. In contrast, the IgG2b isotypes decreased more rapidly. Intranasal immunization with the recombinant BCG strain also induced a DTH response toward BCG antigens at a level similar to that for nonrecombinant BCG. This finding suggests that neither the presence of the heterologous antigen expressed in the bacteria nor the induction of a strong antibody response against the antigen impeded the capacity of the recombinant strain to induce a DTH response. It also suggests that DTH may be considered as a useful marker for i.n. BCG vaccination.

An interesting and surprising observation that resulted from this study is the finding that i.n. administration of BCG producing Sh28GST induced a much stronger immune response against the parasite antigen than i.n. administration with BCG producing Sm28GST. Interestingly, this difference was not observed when the recombinant BCG strains were delivered i.p. Both BCG strains produced the same levels of parasite antigen, and they were recognized equally well by polyclonal antisera. In addition, i.n. immunization with either BCG strain resulted in similar immune responses against BCG antigens. Therefore, Sh28GST appears to be intrinsically more immunogenic than Sm28GST when given by the i.n. route, although the two proteins are approximately 90% identical in the amino acid sequence and highly cross-reactive (28). Furthermore, i.n. immunization with BCG producing Sh28GST resulted in a stronger immune response even against Sm28GST than i.n. immunization with BCG producing Sm28GST, indicating that the two proteins differ in immunogenicity rather than in anti-
genicity. Most of the amino acid substitutions between Sh28GST and Sm28GST lie in the central portion of the protein encompassing the region between residues 115 and 131. It has been demonstrated that peptide 115-131 contains both T- and B-cell recognition sites of Sm28GST in various experimental models (29, 30). Trottet et al. (28) have shown that the lack of cross-reactivity of Sh28GST with the anti-Sm28GST peptide 115-131 antiserum appears to be due to a single amino acid substitution. Construction of new BCG strains producing Sh28GST-Sm28GST hybrid proteins will be helpful to localize the precise difference between the two proteins that is responsible for the difference in immunogenicity. Identification of this structure-function relationship may be useful for understanding the induction of immune responses via the nasal route and perhaps for enhancing such immune responses. The reason for which the difference in immunogenicity is specifically observed after i.n. immunization also warrants further investigation; it may be related to differences in antigen presentation by antigen-presenting cells via the i.n. route compared to the i.p. route of immunization.

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