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# Effective Vaccination of Mice against *Mycobacterium tuberculosis* Infection with a Soluble Mixture of Secreted Mycobacterial Proteins

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**An experimental vaccine that was based on secreted proteins of *Mycobacterium tuberculosis* was investigated in a mouse model of tuberculosis. I used a short-term culture filtrate (ST-CF) containing proteins secreted from actively replicating bacteria grown under defined culture conditions. The immunogenicity of the ST-CF was investigated in combination with different adjuvants, and peak proliferative responses were observed when ST-CF was administered with the surface-active agent dimethyldioctadecylammonium chloride. The immunity induced by this vaccine was dose dependent, and, in the optimal concentration, the vaccine induced a potent T-helper 1 response which efficiently protected the animals against a subsequent challenge with virulent *M. tuberculosis*. Antigenic targets for the T cells generated were mapped by employing narrow-molecular-weight fractions of ST-CF. The experimental vaccine primed a broadly defined T-cell repertoire directed to multiple secreted antigens present in ST-CF. A vaccination with viable *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), in contrast, induced a restricted T-cell reactivity directed to two secreted protein fractions with molecular masses of 5 to 12 and 25 to 35 kDa. The protective efficacy of the ST-CF vaccine was compared with that of a BCG standard vaccine, and both induced a highly significant protection of equal magnitude. The vaccination with ST-CF gave rise to a population of long-lived CD4 cells which could be isolated 22 weeks after the vaccination and could adoptively transfer acquired resistance to T-cell-deficient recipients. My results confirm the hypothesis that *M. tuberculosis* cells release protective antigens during growth. The high efficacy of a subunit vaccine observed in the present study is discussed as a possible alternative to a live recombinant vaccine carrier.**

Human tuberculosis caused by *Mycobacterium tuberculosis* remains a severe global health problem responsible for approximately 8 million new cases and 3 million deaths annually (46). The worldwide incidence of new tuberculosis cases has been falling for the last few decades; however, in recent years, this trend has changed markedly because of the AIDS epidemic and the appearance of multidrug resistant-strains of *M. tuberculosis*.

The only vaccine presently available is *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). However, the efficacy of BCG remains a matter of controversy. BCG generally induces a high level of acquired resistance in animal models of tuberculosis (40). Human trials, however, have demonstrated a varying efficacy, ranging from 80% in some trials (e.g., in the United Kingdom) to a lack of significant protection in several recent trials in developing countries (15). This makes the development of a new and improved vaccine against tuberculosis an urgent matter, and it has been given a very high priority by the World Health Organization. Many attempts to define protective mycobacterial substances have been made, and from 1950 to 1970 several investigators reported the induction of resistance to *M. tuberculosis* in animal models after experimental vaccination with different mycobacterial subfractions (40). This line of research has recently been continued by the demonstration of detectable levels of resistance induced by immunization with culture filtrate proteins (19, 37). However, a specific protective immune response with the potency of BCG has not been achieved by a synthetic vaccine until now. Since increased resistance against challenge with virulent *M. tuberculosis* is generated efficiently only by immunizing with the live bacilli, interest has recently been focused on proteins actively secreted from the live bacilli during growth (1, 5). These proteins are released into the medium of mycobacterial cultures before any significant bacterial autolysis occurs and can be obtained in short-term culture filtrates (ST-CF) (5). CF proteins are major targets for the T-cell response in mice and humans with active tuberculosis (6, 18). Recently, these observations were extended by the important finding that T cells responsible for the recall of protective immunity in a mouse model of tuberculosis are directed to highly purified secreted protein fractions (8). These findings provide the basis for the recently emerging hypothesis that secreted proteins are the key protective antigens leading to an efficient acquired resistance to *M. tuberculosis* (6, 34).

The experimental vaccine investigated in the present study has been based on a defined mixture of secreted CF proteins and an adjuvant, dimethyldeoctadecylammonium chloride (DDA), which is potent for the induction of cell-mediated immunity (42). The monitoring of vaccine potency was supplemented by a detailed characterization of the T cells induced by the vaccination. My study demonstrates that the administration of secreted antigens in an appropriate adjuvant induces a broad spectrum of protective long-lived CD4 cells; this is the first report of a vaccine based on soluble proteins with a protective potency comparable to that of live BCG.

## MATERIALS AND METHODS

**Animals.** These studies were performed with C57BL/6J mice purchased from Bomholtegaard, Ry, Denmark. Female mice, 8

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to 12 weeks old, were used in all experiments. The animals were immunized and kept under good conventional housing conditions for up to 8 months. Two days before the challenge with virulent *M. tuberculosis*, the mice were transferred to biohazard facilities and housed in cages contained within a laminar flow safety enclosure.

**Bacteria.** *M. tuberculosis* H37Rv was grown at 37°C on Löwenstein-Jensen medium or in suspension in Sauton medium enriched with 0.5% sodium pyruvate and 0.5% glucose. BCG Copenhagen was obtained as a freeze-dried vaccine and was rehydrated with diluted Sauton medium followed by a brief treatment in a sonication bath to ensure a disperse suspension.

Killed bacteria to be used as antigen in vitro were produced by incubation overnight in 2% glutaraldehyde as described previously (8).

**Production of ST-CF.** ST-CF was produced as described previously (5). Briefly, *M. tuberculosis* ($8 \times 10^6$ CFU/ml) was grown in enriched Sauton medium at 37°C on an orbital shaker for 7 days. The cultures were centrifuged (12,800 $\times$ *g* for 30 min at 4°C), and the supernatants were filtered with a sterile filter and concentrated 100-fold on an Amicon YM 3 membrane (Amicon, Danvers, Mass.) This preparation will be referred to as ST-CF.

**Fractionation of ST-CF by the multielution technique.** ST-CF protein in a quantity of 5 mg was separated in sodium dodecyl sulfate-10 to 20% polyacrylamide gel electrophoresis (SDS-PAGE) overnight (11-cm-wide center well, 0.75-mm gel) (24). At the end of the electrophoretic run, the gel was trimmed for excess gel and pre-equilibrated in three changes of the electroelution buffer (2 mM sodium phosphate buffer) for 40 min. The multielution was performed as described previously (7). Briefly, gels were transferred to the Multi-Eluter (Kem-En-Tec, Copenhagen, Denmark) and electroeluted (40 V) for 20 min. The protein fractions were aspirated, adjusted to isotonia with concentrated phosphate-buffered saline (PBS), and analyzed by SDS-PAGE by separation on 10 to 20% nonreducing SDS-PAGE gels followed by silver staining (30). Protein concentrations in the fractions were estimated by the bicinchoninic acid method (Pierce, Oud-Beijerland, Netherlands). All fractions were stabilized with 0.5% normal mouse serum and were kept frozen at −80°C until use.

**Preparation of experimental vaccines.** Various amounts of ST-CF (as indicated in Table 1 and Fig. 3) were tested with different adjuvants. DDA (Eastman Kodak, Rochester, N.Y.) was suspended in distilled water (2.5 mg ml$^{-1}$), and a homogeneous dispersion of the powder was obtained by heating the suspension to 80°C for 5 to 10 min. After cooling at room temperature, the suspension was mixed with PBS or diluted ST-CF. The mice were injected subcutaneously (s.c.) with 250 μg of DDA in a volume of 0.2 ml.

Liposomes were prepared as described previously (21). Briefly, small lamellar vesicles were prepared by bath sonication (20°C) of equimolar phosphatidylcholindistearoyl (200 μmol) and cholesterol in 10 ml of distilled water. This was followed by probe sonication at 60°C for 15 min. After cooling to room temperature, 0.5 mg of monophosphoryl lipid A (MPL) was added to each milliliter of liposome suspension, together with either ST-CF or PBS, and entrapment was obtained by dehydration-rehydration. Between 60 and 70% of the added ST-CF was entrapped in the liposomes. Mice were injected intravenously (i.v.) with 0.1 ml of the liposome suspension.

RIBI adjuvant was prepared by dissolving MPL and trehalose dimycolate (TDM) (RIBI; Immunochem. Research Inc., Hamilton, Mont.) in a mixture of chloroform and methanol (4/1). The solvent was evaporated, and the components were ground with incomplete Freund's adjuvant. ST-CF was added in 0.2% Tween saline. Each injection contained 50 μg of MPL, 50 μg of TDM, and ST-CF in a volume of 0.2 ml. The vaccines with RIBI adjuvant were injected s.c.

**Vaccination challenge and necropsy.** The mice were immunized with $5 \times 10^4$ CFU of BCG s.c. in 0.2 ml of saline at the base of their tails. The mice were rested for 12 to 14 weeks and were then challenged by an i.v. injection of $5 \times 10^4$ CFU of *M. tuberculosis*. The course of disease was compared with that for a corresponding group of unimmunized mice during a period of 28 days. The challenge necropsy interval that yielded the largest difference in the numbers of bacteria recovered from the spleens of these two groups of mice (the largest protective window) was used for the subsequent testing of experimental vaccines. The mice were immunized with the experimental vaccines three times at weekly intervals. In the first experiment, the mice were left for 12 to 14 weeks after the first injection and were then challenged by an i.v. injection of $5 \times 10^4$ viable *M. tuberculosis*. On the basis of the study of BCG-vaccinated mice (see Results), the bacteria were enumerated 2 weeks after challenge. In the second series of protection experiments, the mice were challenged after 5 to 6 weeks by an intraperitoneal injection of $10^6$ *M. tuberculosis*. Enumerations of bacteria in the organs of infected mice were done by plating double serial 10-fold dilutions of organ homogenates on Löwenstein-Jensen medium. Colonies were counted after 3 to 4 weeks of incubation, and the data were expressed as the log 10 values of the geometric means of counts obtained with 6 to 12 mice. All treatments were coded, and the results were not evaluated until all data had been collected.

**Adoptive transfer experiments.** C57BL/6j mice were rendered T-cell deficient by whole-body gamma ray irradiation (500 rad) as described previously (35). At 24 hours after irradiation, the mice received one spleen equivalent of CD4 or CD8 cells purified by negative selection using murine T-cell subset columns (British Biotechnology, Oxon, United Kingdom). The donor mice had been rested for 22 weeks after the initial vaccination. Two hours after the adoptive transfer, the mice were challenged with $5 \times 10^4$ *M. tuberculosis* i.v. The animals were killed 14 days later, and the spleens and livers were removed for bacterial enumeration.

**Lymphocyte cultures.** Lymphocytes were obtained by preparing single-cell suspensions from spleens of mice 2 to 3 weeks after the previous immunization. The establishment of lymphocyte cultures has been described previously (6). Briefly, ST-CF or antigenic fractions thereof were added to microcultures containing $2 \times 10^5$ lymphocytes in a volume of 200 μl of RPMI 1640 supplemented with $5 \times 10^{-5}$ M 2-mercaptoethanol–penicillin–streptomycin–1 mM glutamine and 0.5% (vol/vol) fresh mouse serum. ST-CF was tested in various concentrations; killed bacteria were used at a concentration of $2 \times 10^6$ cells per ml, while ST-CF fractions were used at 2 μg/ml. Cellular proliferation was investigated by pulsing cultures (1 μCi of [$^3$H]thymidine per well) after 48 h of incubation and then further incubating for 22 h, before the plates were harvested and processed for liquid scintillation counting (Beta counter; LKB).

Culture supernatants were harvested from parallel cultures after 24 h of incubation (for the determination of interleukin-2 [IL-2], IL-4, and IL-5) and after 48 h (for the determination of gamma interferon [IFN-γ], IL-6, and granulocyte macrophage–colony-stimulating factor [GM-CSF]).

To characterize the responding T-cell subset, the T-cell coreceptors CD4 and CD8 were blocked by diluting the monoclonal antibodies (MAbs) anti-CD4 (clone Gk 1.5) and

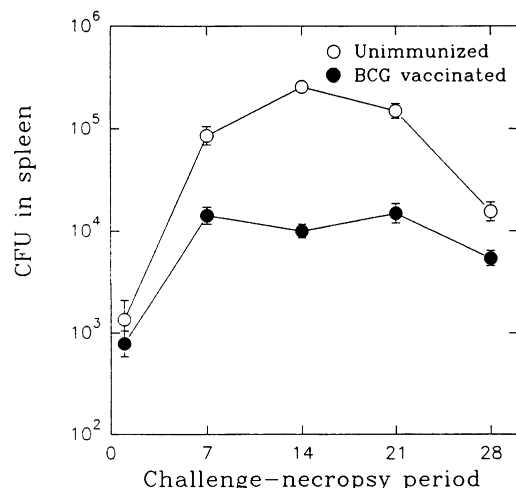


FIG. 1. Course of infection with *M. tuberculosis* in unimmunized and BCG-vaccinated C57BL/6J mice. C57BL/6J mice were infected with $5 \times 10^4$ viable units of *M. tuberculosis*, and the growth of the bacteria in the spleens was investigated over time. The CFU per spleen values indicated are geometric means ± SEM ($n = 6$). At day 14, an exceedingly low SEM hides the error bar behind the symbol.

anti-CD8 (clone 53-6.7) directly in the cultures before incubation. The restriction element was similarly determined by blocking with anti-$I\text{-}A^b$ (Tib 120) or anti-$H\text{-}2D^b$ (Pharmingen, San Diego, Calif.). All dilutions of MAbs were based on previous titrations.

**Cytokine assays.** The amounts of cytokines present in culture supernatants were quantified by commercially available enzyme-linked immunosorbent assay (ELISA) kits. For the determination of IFN-γ (Holland Biotechnology, Leiden, the Netherlands), values of less than 50 pg/ml were considered negative. For determination of IL-2, IL-4, IL-5, IL-6, and GM-CSF (Endogen, Boston, Mass.), values of less than 20 pg were considered negative.

**Antibody titers to ST-CF of different IgG isotypes.** Serum samples obtained from the mice 5 weeks after the first immunization were analyzed in twofold dilutions on ELISA plates coated with ST-CF (100 μl per well; 5 μg/ml). The plates were incubated for 2 h at room temperature, and, after washing, the plates received peroxidase-labeled goat antimouse immunoglobulin G (IgG), IgG2A (1/1,000), or IgG1 (1/2,000) (Serotec, Kidlington, Oxford, United Kingdom). Antibody titers to ST-CF of different isotypes are expressed as the dilutions that gave an absorbency of 1 (IgG1) or 0.4 (IgG2A). The titers have been adjusted for different levels of specific IgG induced by the vaccines and are therefore presented as relative isotype titers.

**Statistical methods.** The efficacies of different vaccination protocols have been compared by one-way analysis of variance of log 10 CFU. Standard errors of the means (SEM) have been given for all figures (for the experiment presented in Fig. 3C, data are given as a pooled SEM).

## RESULTS

**BCG-induced acquired resistance to infection with *M. tuberculosis*.** In experimental animals, immunization with BCG efficiently induces protective immunity against tuberculosis (40). In the present study, BCG Copenhagen was used as a reference vaccine for the establishment of an animal model suited for comparative studies of protective immunity.

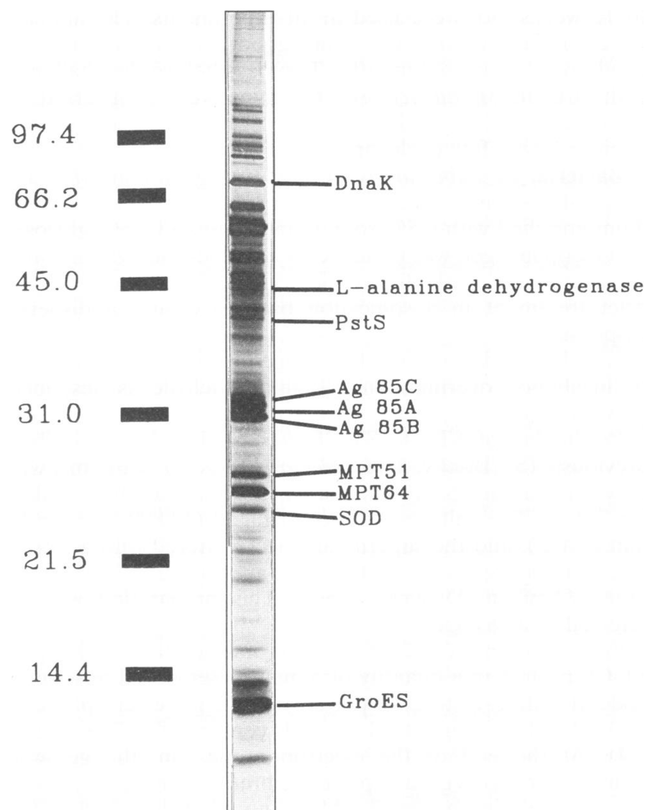


FIG. 2. The components present in a defined ST-CF harvested from a logarithmically growing culture of *M. tuberculosis*. The filtrate was separated in SDS-PAGE and silver stained. Previously characterized major secreted proteins are indicated on the right side of the lane, while the migration of molecular weight markers is indicated on the left side. For a comprehensive review of defined mycobacterial antigens, see reference 51.

C57BL/6J mice were vaccinated with BCG and left for 12 to 14 weeks to ensure that the nonspecific inflammatory response to the vaccine itself had ceased before the administration of a virulent challenge. Vaccinated and naive mice received $5 \times 10^4$ *M. tuberculosis* CFU intravenously, and the growth of the bacteria in the spleens was observed during a period of 28 days (Fig. 1). The bacteria multiplied rapidly in unimmunized mice and reached a maximal level of $3 \times 10^5$ CFU at day 14. After this time, the bacteria were gradually eliminated, and their numbers reached $2 \times 10^4$ at day 28. In vaccinated mice, only limited growth of *M. tuberculosis* was observed, and the difference in the numbers CFU found in vaccinated and naive mice (the protective window) was maximal (a difference of 30 times) at day 14. This experiment was repeated twice with the same result, and a 14-day challenge necropsy interval was chosen for the subsequent testing of experimental vaccines.

**An experimental vaccine based on secreted mycobacterial proteins.** ST-CF harvested from mid-logarithmic-phase cultures of *M. tuberculosis* contains high concentrations of previously characterized major secreted proteins and several undefined protein bands (5) (Fig. 2). This mixture of secreted proteins has in the present study been chosen as the basis for an experimental vaccine.

Pilot experiments demonstrated soluble mixtures of mycobacterial proteins to be weakly immunogenic, since only very limited responses (proliferation and IFN-γ production) could

TABLE 1. Proliferative responses to mycobacterial antigens after immunization with ST-CF in different adjuvant combinations

| Expt | Immunization[a] | Proliferation with the following antigens[b]: | |
|---|---|---|---|
| | | Killed bacteria | ST-CF |
| 1 | | | |
| | PBS | 2.1 ± 0.2 | 5.0 ± 0.4 |
| | ST-CF-PBS | 1.5 ± 0.1 | 6.0 ± 1.1 |
| | RIBI | 2.1 ± 0.3 | 5.8 ± 0.8 |
| | ST-CF-RIBI | 5.1 ± 0.4 | 17.5 ± 0.1 |
| | DDA | 1.8 ± 0.2 | 5.0 ± 0.4 |
| | ST-CF-DDA | 10.3 ± 1.0 | 31.0 ± 3.0 |
| 2 | | | |
| | PBS | 3.7 ± 0.9 | 5.3 ± 0.6 |
| | ST-CF-PBS | 3.1 ± 0.4 | 5.5 ± 0.6 |
| | Liposomes | 3.2 ± 0.6 | 5.1 ± 1.1 |
| | ST-CF-liposomes | 3.7 ± 0.6 | 7.8 ± 1.0 |
| | DDA | 1.3 ± 0.1 | 2.7 ± 0.5 |
| | ST-CF-DDA | 5.9 ± 1.1 | 14.6 ± 1.0 |

[a] In experiment 1, mice were immunized s.c. three times with 100 μg of ST-CF or the respective control preparations. In experiment 2, mice were immunized twice with 250 μg of ST-CF either i.v. (liposomes) or s.c. (DDA).

[b] Proliferation values are expressed as mean counts per minute ($10^3$) ± SEM of results obtained for three to five mice in each group.

be measured when lymphocytes isolated from animals primed s.c. with ST-CF were restimulated in vitro (results not shown). Therefore, it was decided to evaluate the effect of different adjuvants previously reported to induce cell-mediated immunity. RIBI adjuvant contains the microbial immunostimulants TDM and MPL, and both this adjuvant and DDA have been reported to induce predominantly cell-mediated immunity (23, 42). Liposomes have been used for a variety of purposes and have in the present study been supplemented with MPL, a synergistic combination previously reported to possess marked adjuvanticity (17). ST-CF was tested in combination with these adjuvants in two independent experiments. Mice were immunized with the various antigen-adjuvant combinations, and the systemic immunity induced was evaluated using isolated spleen lymphocytes 2 to 3 weeks after the last immunization. The lymphocytes were stimulated with mycobacterial antigen preparations in vitro, and proliferative responses were monitored (Table 1). The adjuvant DDA promoted peak proliferative responses to the mycobacterial antigens in both experiments. The adjuvanticity was not associated with nonspecific stimulation, since a low level of proliferation was observed in the control group receiving the adjuvant alone. DDA was therefore chosen for further immunization studies.

The optimal concentration of ST-CF was determined by immunizing mice with a range of doses from 10 to 250 μg. Spleen lymphocytes of immunized mice were restimulated in vitro with ST-CF, and cellular proliferation and IFN-γ release were investigated. Mice immunized with doses of ST-CF from 10 to 100 μg exhibited increasing proliferative responses (Fig. 3A), whereas increasing the dose to 250 μg provided no additional boosting of the proliferative response. IFN-γ secretion demonstrated a markedly different dose response relationship. Doses of 10, 30, and 100 μg induced comparable high levels of IFN-γ, whereas an increase to 250 μg resulted in a pronounced drop in the secretion of IFN-γ. The relative levels of specific IgG1 and IgG2A induced by the different doses of ST-CF were used to give an indirect indication of the lymphocyte subsets involved (32). Increasing doses of ST-CF were found to shift the relative contributions of the isotypes toward IgG1, leaving the levels of IgG2A largely unchanged (Fig. 3B).

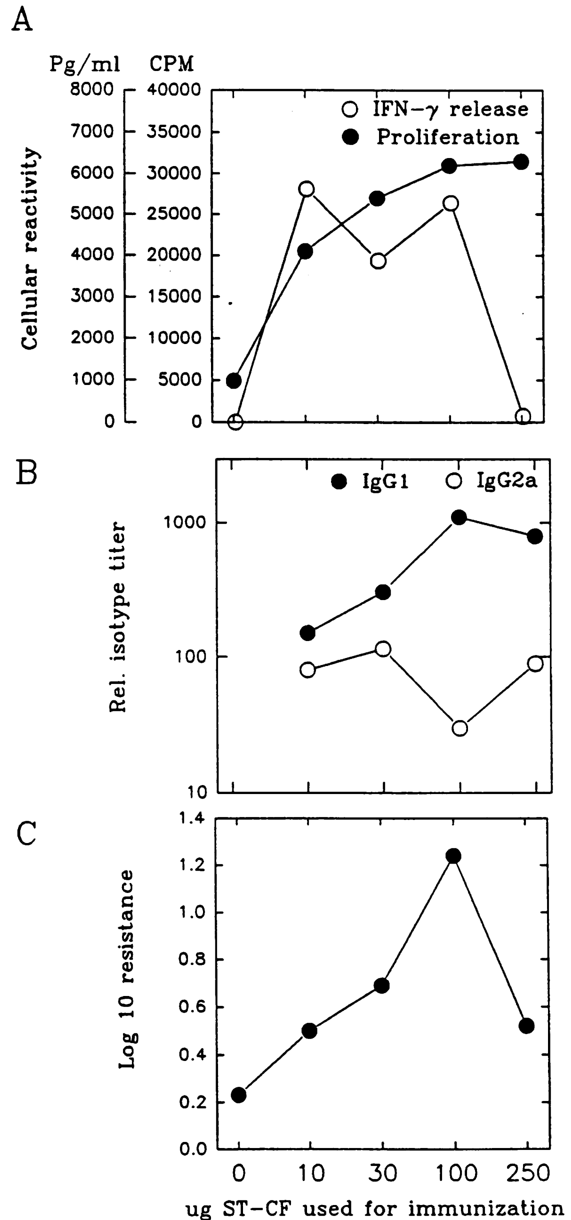


FIG. 3. Immune reactions after immunization with different doses of ST-CF in DDA. C57BL/6J mice were immunized three times with the experimental vaccines, and cellular reactivities (A) and antibody levels (B) were monitored 3 weeks after the last injection. The responses depicted were obtained with cells or serum pooled for groups of five mice. SEM are not indicated but are always below 20%. Six mice for each dose of ST-CF were rested for 10 to 12 weeks before they received an i.v. challenge of virulent *M. tuberculosis*. The log 10 difference between numbers of bacteria harvested from the spleens of control mice and from those of vaccinated mice (log 10 resistance) was investigated 2 weeks after challenge (C). The log 10 values indicated are geometric means, and the pooled SEM is 26% of the means. The monitoring of resistance was repeated twice, with the same overall result.

The remaining animals (six mice for each dose of ST-CF) were left for an additional 12-week period before they received an i.v. challenge with $5 \times 10^4$ virulent *M. tuberculosis* (Fig. 3C). A 14-day challenge necropsy interval was chosen, and the

TABLE 2. Proliferative responses by lymphocytes isolated from ST-CF-DDA-vaccinated mice

| Stimulant | Proliferative response with the following blocking reagents[a,b]: | | | | |
|---|---|---|---|---|---|
| | None | Anti-CD4 | Anti-CD8 | Anti-*I-A* | Anti-*H-2D* |
| Medium alone | 0.46 ± 0.04 | 1.24 ± 0.07 | 1.76 ± 0.17 | 2.44 ± 0.14 | 3.45 ± 0.02 |
| ST-CF (0.2 μg/ml) | 9.45 ± 0.45 | 2.23 ± 0.09 | 9.02 ± 0.96 | 1.52 ± 0.04 | 9.29 ± 0.90 |
| ST-CF (2 μg/ml) | 24.22 ± 0.65 | 5.52 ± 0.09 | 21.23 ± 1.29 | 2.00 ± 0.38 | 26.03 ± 0.59 |
| Concanavalin A | 38.94 ± 0.48 | 20.63 ± 2.24 | 17.18 ± 1.42 | 25.16 ± 1.05 | 40.27 ± 1.70 |

[a] Proliferation values are expressed as mean counts per minute ($10^3$) ± SEM of triplicate analyses performed on cells pooled from five mice.
[b] Proliferative responses were blocked by adding MAbs directly to the cell cultures.

numbers of bacteria in the spleens were determined. All groups of mice immunized with ST-CF demonstrated a significant level of protective immunity; however, the highest level was obtained when mice were immunized with 100 μg of ST-CF, whereas increasing the dose to 250 μg resulted in a decrease in the level of resistance. This experiment was repeated twice with the same general result. Thus, all further studies were done with experimental vaccines containing 100 μg of ST-CF in DDA.

**Characterization of the immune response induced by immunization with secreted proteins.** Mice immunized with 100 μg of ST-CF in DDA exhibited an optimal response, as judged by high levels of acquired resistance and a powerful cellular response to ST-CF. To characterize the cells mediating this response, the T-cell coreceptors CD4 and CD8 were blocked by MAbs. The major histocompatibility complex restriction element used was similarly determined by blocking either *I-A* or *H-2D* with MAbs directed to these surface elements. The vaccinated mice were found to exhibit a powerful dose-dependent proliferative response to ST-CF that could be blocked by either anti-CD4 or anti I-A (Table 2), thus demonstrating that the response induced by the vaccination was a classical major histocompatibility complex class II-restricted CD4 T-cell response. To further characterize the T cells primed by the vaccination, supernatants from in vitro lymphocyte cultures were tested for the presence of various cytokines (Table 3). No cytokines could be detected in unstimulated cultures, whereas high levels of IL-2, IFN-γ, IL-6, and GM-CSF were detected in cultures stimulated with ST-CF. IL-4 and IL-5 were synthesized in exceedingly low amounts, indicative of activity skewed toward a T-helper 1 (Th-1) type of response.

**The specificity of the T-cell response in mice immunized with secreted proteins or BCG.** The specificity of the T-cell response induced by the experimental vaccination was compared with the response found after immunization with BCG. Mice were immunized with BCG or ST-CF-DDA, and 5 weeks after the first injection, the animals were sacrificed and the systemic cell-mediated immunity was evaluated by stimulating splenic T cells with protein fractions obtained by the multi-elution technique (7). This technique enables the separation of complex protein mixtures into narrow-molecular-mass fractions suited for specificity analyses in T-cell cultures. The technique was employed on ST-CF and resulted in 14 protein fractions with a minimal overlap between neighboring fractions, corresponding closely to results previously obtained by this method (7). The fractions were adjusted to the same protein concentration and used to stimulate spleen lymphocytes obtained from vaccinated mice.

Mice immunized with ST-CF-DDA exhibited a pronounced cellular reactivity to all components of ST-CF, as demonstrated by both a powerful proliferation and a marked release of IFN-γ (Fig. 4). The highest release of IFN-γ was elicited by antigens with molecular masses ranging from 70 to 80 kDa; however, the vaccine had primed an exceedingly broad T-cell repertoire recognizing an extensive spectrum of secreted antigen fractions. An immunization with live BCG, in contrast, triggered only a narrow part of the T-cell repertoire and were predominantly found to stimulate IFN-γ-producing T cells directed to secreted antigens with molecular masses of 5 to 12 and 25 to 35 kDa. Cells from unimmunized mice gave a marginal proliferative response to fraction 6 (26 to 30 kDa), but without detectable release of IFN-γ.

**Vaccination with secreted proteins evokes a highly efficient acquired resistance mediated by long-lived CD4 cells.** The relative potency of the experimental vaccine was investigated in two independent experiments by testing its protective efficacy in parallel with that of a BCG standard vaccine. Experiment 1 followed the same schedule as previous experiments and applied an i.v. challenge, whereas the challenge was administered intraperitoneally in experiment 2. In experiment 1, immunization with ST-CF reduced the number of CFU in the spleen by 1.24 log, whereas a BCG immunization reduced the number of CFU by 1.41 log (Table 4). These differences between immunized and unimmunized mice were highly significant ($P = 0.0001$), whereas no significant difference was found between immunization with BCG and ST-CF-DDA. Neither ST-CF-PBS nor DDA alone induced any significant protection. The same result was obtained when the lungs were investigated, although the differences in the numbers of CFU found in control and vaccinated animals were less than the differences found in the spleens. Experiment 2 was confirmatory and led to the conclusion that both BCG vaccination and immunization with ST-CF give rise to highly significant levels of acquired resistance to *M. tuberculosis*.

Specific long-term immunity is the central goal of any rational vaccination strategy. To confirm that long-lived T cells

TABLE 3. Cytokine release by lymphocytes isolated from ST-CF-DDA-vaccinated mice.

| Stimulant | Cytokine release (pg/ml)[a] | | | | | |
|---|---|---|---|---|---|---|
| | IL-2 | IFN-γ | IL-4 | IL-5 | IL-6 | GM-CSF |
| Medium alone | <20 | <50 | <20 | <20 | <20 | <20 |
| ST-CF (2 μg/ml) | 1,030 ± 44 | 5,268 ± 1,365 | 35 ± 5 | <20 | 3,025 ± 211 | 656 ± 42 |

[a] Cytokine data are means ± SEM of triplicate analyses of cell culture supernatants pooled from five mice.

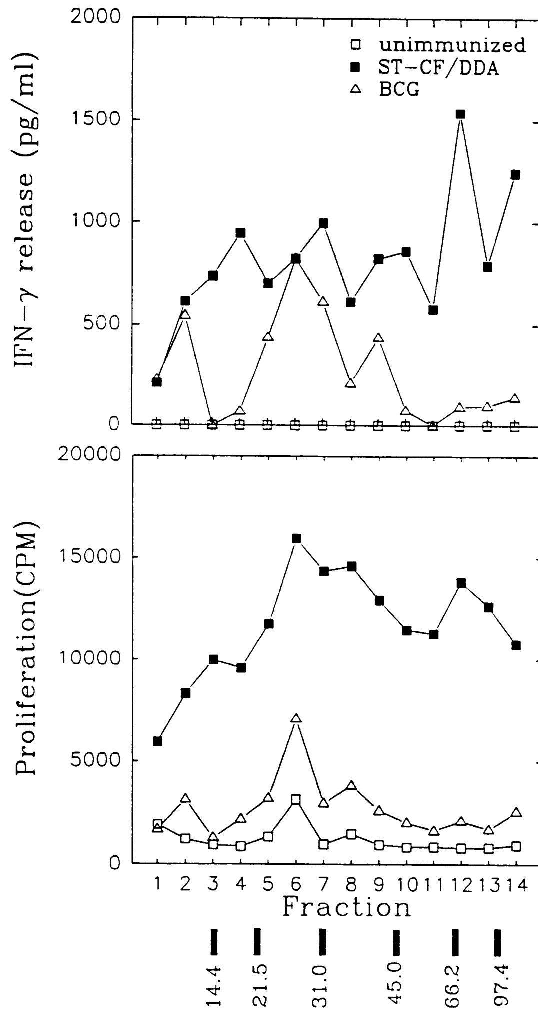


FIG. 4. Cellular responses of lymphocytes isolated from vaccinated mice. Mice were immunized with either BCG or ST-CF in DDA. Spleen lymphocytes were isolated at day 35 and stimulated in vitro with a panel of narrow-molecular-weight fractions of ST-CF. The top panel shows quantities of IFN-γ released to the culture supernatants, and the bottom panel shows cellular proliferation in the cultures. All responses depicted are means ($n$ = 5), and the pooled SEM are 25.7% (IFN-γ release) and 17.7% (proliferation), respectively. The experiment was repeated three times with the same result.

were responsible for the acquired resistance observed, groups of vaccinated mice and control animals were rested for 22 weeks after vaccination. We then investigated the capacity of splenic T-cell subsets harvested from vaccinated mice to adoptively protect irradiated T-cell-deficient recipients against a subsequent challenge infection. The group of mice that received CD4 cells from donors immunized with the ST-CF vaccine demonstrated a highly significant reduction in bacterial numbers (1.34 log), whereas cells derived from BCG-vaccinated animals reduced the bacterial numbers by 0.84 log (Table 5). None of the treatments induced CD8 cells with protective capacity.

## DISCUSSION

A striking characteristic of immunity to the intracellular pathogens *M. tuberculosis* and *Listeria monocytogenes* is the superior ability of live vaccines to induce a specific protective immune response (26, 33, 49). This classical observation is the basis for two important new approaches in tuberculosis vaccine development: (i) cloning of immunologically relevant antigens from *M. tuberculosis* into a live recombinant carrier, e.g., BCG (20, 41), and (ii) investigation of antigens secreted from the live bacteria during growth as a possible source of protective antigens (1, 5). The present study is based on the latter strategy and demonstrates that immunization with a mixture of secreted proteins combined with an appropriate adjuvant induces a specific cellular immune response and long-term protection of high potency. To our knowledge, this is the first study to demonstrate a protective immune response induced by a mixture of soluble proteins with a potency equal to that of live BCG. The data presented herein together with other recent immunization studies using CF proteins (19, 37) strongly support the hypothesis that specific T cells involved in protective immunity to *M. tuberculosis* are directed toward secreted antigens (6, 34).

Several studies have investigated the capacity of particulate cell wall-derived antigen preparations to induce a protective immune response in animal models of tuberculosis (25, 47, 48). Ribi et al. conducted a series of experiments in which BCG cell walls treated with a light mineral oil were found to induce protective immunity superior to that obtained with viable BCG (25, 38). In a subsequent study, however, it was demonstrated that resistance peaked shortly after immunization (3) and waned when the mice were left for prolonged periods of time, suggesting a mixed reaction mediated by both specific and

TABLE 4. Bacterial numbers in organs of vaccinated mice after challenge with virulent *M. tuberculosis*

| Immunization[a] | Result for expt 1[b] | | Result for expt 2[b] |
|---|---|---|---|
| | Spleen | Lung | Spleen |
| Control | 5.41 | 3.61 | 4.83 |
| BCG | 4.00 ($P$ = 0.0001) | 2.94 ($P$ = 0.0012) | 2.96 ($P$ = 0.0002) |
| ST-CF-PBS | 5.48 | 3.70 | ND |
| PBS-DDA | 5.18 | 4.03 | 4.38 |
| ST-CF-DDA | 4.17 ($P$ = 0.0001) | 2.93 ($P$ = 0.0026) | 3.46 ($P$ = 0.0042) |

[a] Mice were immunized with BCG or injected three times with the experimental vaccines.

[b] Bacterial numbers are expressed as the log 10 values of the geometric means ($n$ = 6 in experiment 1 and $n$ = 12 in experiment 2). SEM are less than 0.16 in experiment 1 and less than 0.32 in experiment 2. $P$ values have been given for bacterial numbers that are significantly different from the numbers found for unimmunized control animals.

TABLE 5. Bacterial numbers in the spleens of recipient mice reconstituted with T-cell subsets from vaccinated mice[a]

| Immunization of donor mice[b] | Bacterial number[c] with the following subsets transferred: | |
|---|---|---|
| | CD4 | CD8 |
| Control | 5.24 | 5.66 |
| BCG | 4.40[d] | 5.29 |
| PBS-DDA | 5.34 | 5.43 |
| ST-CF-DDA | 3.90[d] | 5.24 |

[a] Recipient mice were syngeneic whole-body irradiated mice (500 rad). Each recipient mouse received one spleen equivalent of purified CD4 or CD8 cells.

[b] Donor mice were immunized 22 weeks before the transfer was done.

[c] Bacterial numbers are the log 10 values of geometric means based on duplicate analyses of three animals for each treatment. The pooled SEM for the experiment was 0.21.

[d] Values significantly different from bacterial numbers in control mice.

nonspecific immune mechanisms. The following demonstration of high levels of nonspecific resistance induced by the mycobacterial derivatives trehalose dimycolate and muramyl dipeptide (28), emphasized the importance of testing experimental tuberculosis vaccines without the interference imposed by immunostimulating components of the mycobacterial cell wall. This concern was fundamental for the planning of the present experiments, in which soluble secreted proteins devoid of inflammatory cell wall components were used for immunization in combination with the mild adjuvant DDA. DDA has been extensively tested in a variety of laboratory animals (42, 43, 50) and has also been tried in human tetanus toxoid vaccines (44). These studies have consistently found that DDA favors strong cell-mediated immune reactions, as measured by delayed-type hypersensitivity reactions, without some of the major unacceptable side effects caused by other potent adjuvants (local ulceration or toxicity). The present study fully supports this idea; DDA was found to promote a marked induction of cell-mediated immunity to ST-CF without side effects and without enhancement of nonspecific resistance.

Murine CD4 T cells consist of at least two distinct subsets (31). Th-1 cells secrete IFN-$\gamma$ and IL-2 and mediate predominantly delayed-type hypersensitivity, whereas Th-2 cells are characterized by the secretion of IL-4 and IL-5, leading to antibody production. Th-1 cells are generally believed to be responsible for activation of macrophages, leading to protective immunity against intracellular pathogens. A predominant Th-2 response, in contrast, may exacerbate the disease, in analogy with the situation found in leishmania-infected mice (39). It is a broadly recognized phenomenon that adjuvants possess a selectivity for the induction of a particular class of immune responses. The general finding has been that adjuvants containing microbial derivatives (mycobacteria or corynebacteria) promote powerful delayed-type hypersensitivity reactions, whereas the depot adjuvants (aluminum hydroxide or Freund's incomplete adjuvant) are efficient inducers of a humoral response (10, 11). Recently, however, a more differentiated picture has emerged on the basis of the finding that the levels of IgG1 and IgG2A are controlled by Th-2 and Th-1 cells, respectively (32). This knowledge has recently been used to demonstrate adjuvant control of T-cell subset induction, leading to changes in antibody isotopes as well as cytokine production (12, 22). The present study demonstrates that DDA promotes the triggering of major histocompatibility complex class II-restricted, CD4 T cells skewed toward the Th-1 type, as judged by the very pronounced production of IFN-$\gamma$ and IL-2. However, what is of importance is that this study pinpoints the influence of antigen dose on vaccine efficacy and emphasizes the necessity of careful dose response investigations when experimental vaccines are tested. Increases in the doses of the immunogen from 10 to 100 $\mu$g were in the present study associated with increased levels of IgG1, indicating an enhanced contribution by Th-2 cells, and in agreement with this finding was the dose-dependent increase in cellular proliferation not associated with a corresponding increase in IFN-$\gamma$ production (Fig. 3A). A shift toward a Th-2 type of immune response induced by high doses of antigen has previously been reported with different doses of *Leishmania major* (14). On the basis of these findings, Bretscher has recently proposed a low-dose immunization strategy to improve the efficacy of vaccination against tuberculosis (13). Our results are in agreement with this hypothesis but imply that a killed vaccine presents an even more delicate balance; even though the lowest dose of ST-CF led to a predominant Th-1 response associated with low levels of IgG1, a dose 10 times as high was needed for the induction of efficient resistance.

Vaccination should at best leave a stable specific immunological imprint that enables the host to mount an accelerated and efficient protective immune response at a given time later in life. This immune surveillance is conducted by memory T cells, which patrol the peripheral tissues for invading pathogens (27). We have previously demonstrated that the antigenic targets for memory effector T cells that mediate long-lived immunity to *M. tuberculosis* are secreted antigens (8). The present study supports this observation and provides substantial evidence to support the view that an adequate immune response against *M. tuberculosis* can be induced without the need for the live bacilli.

In the present study, the fine specificity of the protective T-cell response was investigated. The experimental vaccine was found to prime a broad T-cell response directed to most of the components in ST-CF. A BCG vaccination, on the other hand, was found to induce a selective T-cell response toward molecules in the molecular mass region 6 to 12 and 25 to 35 kDa. Secreted antigens within these regions have previously been identified as key antigenic targets responsible for a marked release of IFN-$\gamma$ in a model of the natural infection with *M. tuberculosis* established in mice (4). Taken together, these findings may suggest that a limited selection of secreted mycobacterial antigens is responsible for induction of protective immunity, and we are currently investigating the resistance induced by such purified antigen fractions in our animal model.

The level of acquired resistance provided by human BCG vaccination has been found to vary considerably in different field trials (15), although no evidence for efficacy differences among the various BCG strains could be demonstrated (29). An explanation for this variation should possibly be sought in the fact that the capacity of a live vaccine to induce a protective immune response against *M. tuberculosis* and *L. monocytogenes* depends on an initial multiplication of the microorganism (9, 36). The efficacy of the vaccine can therefore be abrogated by the simultaneous administration of isoniazid that efficiently arrests bacterial growth (45). The multiplication of BCG in a vaccinated human population may be expected to vary considerably because of two major factors: (i) genetically determined differences in the natural resistance of human populations, in analogy with the differences observed in different mouse strains (16), and (ii) sensitization with environmental mycobacteria which influences the prevaccination level of acquired resistance (2). To avoid the dependency of initial bacterial multiplication, a subunit vaccine based on a selection of protective antigens may provide a feasible alternative to a live recombinant carrier. If successful, such an approach may reduce the large variation found in the protective efficacy of BCG programs in different areas of the world.

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