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Enhancement of Innate and Cell-Mediated Immunity by Antimycobacterial Antibodies

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We investigated the ability of human antibodies induced by *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccination to protect against mycobacterial infections. Serum samples containing mycobacterium-specific antibodies were obtained from volunteers who had received two intradermal BCG vaccinations 6 months apart. Significant increases in lipoarabinomannan (LAM)-specific immunoglobulin G (IgG) were detected after both the primary and booster vaccinations. Effects of mycobacterium-specific antibodies on surface binding and internalization of BCG by neutrophils and monocytes/macrophages were studied, using green fluorescent protein (gfp)-expressing BCG. Surface-bound gfp-expressing BCG were distinguished from intracellular BCG by surface labeling with LAM-specific monoclonal antibody. Internalization of BCG by phagocytic cells was shown to be significantly enhanced in postvaccination serum samples. Furthermore, the inhibitory effects of neutrophils and monocytes/macrophages on mycobacterial growth were significantly enhanced by BCG-induced antibodies. The growth-inhibiting effects of postvaccination sera were reversed by preabsorption of IgG with Protein G. Finally, the helper effects of antimycobacterial antibodies for the induction of cell-mediated immune responses were investigated. BCG-induced antibodies significantly enhanced proliferation and gamma interferon production in mycobacterium-specific $CD4^+$ and $CD8^+$ T cells, as well as the proportion of proliferating and degranulating $CD8^+$ T cells. We conclude that mycobacterium-specific antibodies are capable of enhancing both innate and cell-mediated immune responses to mycobacteria.

It is estimated that a third of the world's population is infected with *Mycobacterium tuberculosis*, and annually there are about nine million new cases of clinical tuberculosis (TB) (8). The live attenuated *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine has been used extensively in an attempt to prevent TB, but its efficacy is limited. A meta-analysis of the numerous trials with BCG concluded that BCG confers about 80% protective efficacy against disseminated disease but only a median of 50% protection against pulmonary TB (6). Considering the incomplete protection conferred by BCG, one of the highest priorities in TB research is the development of a new vaccine with greater efficacy than BCG. A better understanding of the immune mechanisms protective against mycobacteria would be useful for the development of such a new vaccine. It is generally agreed that a strong cell-mediated immune response is essential for protection against mycobacteria. However, the innate and humoral elements of the immune system may have important adjunctive roles.

Previously we demonstrated that intradermal BCG vaccination induces antibodies of the immunoglobulin G_1 (IgG_1), IgG_2, and IgG_3 isotypes (11). An important target of the antibody responses induced by intradermal BCG vaccination was found to be lipoarabinomannan (LAM), a major component of the mycobacterial cell wall (3). Several studies suggest that anti-LAM antibodies may have an important protective role. Passively administered monoclonal antiarabinomannan antibody increased the survival of mice after challenge with *M. tuberculosis* (22). Another study showed that antibodies induced by vaccination with arabinomannan-protein conjugates were partially protective in experimentally infected animals (10). Furthermore, an inverse correlation between the titer of LAM-specific antibodies and the risk of disseminated disease has been reported for human beings (7).

Earlier we also demonstrated that BCG could induce secretory mycobacterium-specific antibodies (3). Oral vaccination with BCG indeed induced a significant increase in anti-LAM-specific IgA. This is important, as antimycobacterial antibodies could play a role not only in systemic immunity, but also in mucosal protection. Intranasal administration of mycobacterium-specific IgA significantly reduced the bacterial load in the lungs of mice after aerosol challenge with *M. tuberculosis* (25).

In the context of infections with other microorganisms it has been shown that antibodies could enhance immunity through many mechanisms, including neutralization of toxins, opsonization, activation of complement, promotion of cytokine release, enhanced antibody-dependent cellular cytotoxicity, and enhanced antigen presentation. In this study we investigated whether human antimycobacterial antibodies induced by BCG vaccination have any protective effects and by which mechanisms such protective immunity is enhanced. We found that sera containing mycobacterium-specific antibodies enhance both innate and adaptive cellular immune mechanisms.

MATERIALS AND METHODS

Vaccination. Ten purified protein derivative (PPD)-negative volunteers were recruited to receive two intradermal vaccinations 6 months apart, as described previously (12). All 10 volunteers received a primary vaccination of 3×10^6 CFU of the Connaught strain of BCG intradermally. Eight of the 10 volunteers were given identical booster vaccinations 6 months later. Serum samples from these

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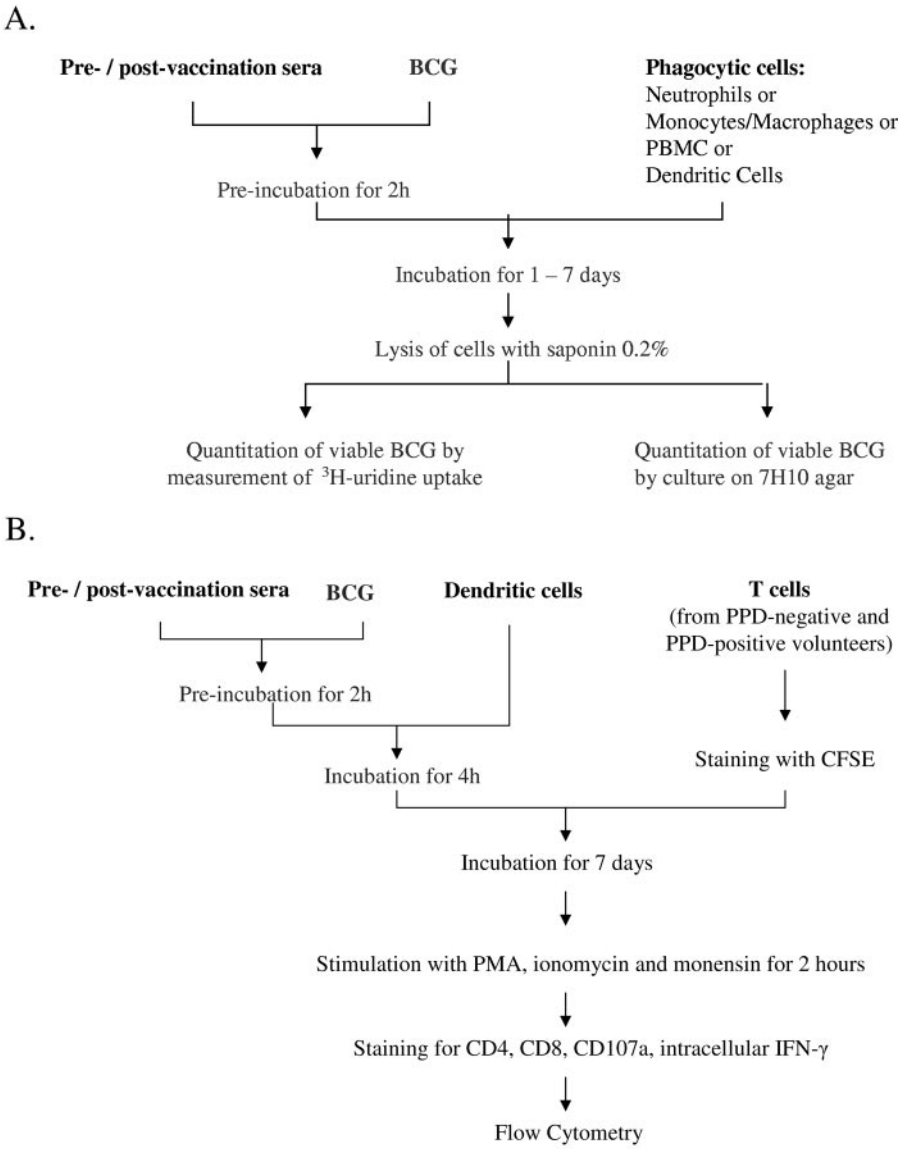

FIG. 1. Experimental designs to test growth-inhibiting effects of mycobacterium-specific IgG in conjunction with phagocytic cells (panel A) and to test enhancement of cell-mediated immunity by mycobacterium-specific IgG (panel B).

volunteers were obtained on days 0, 56, 112, 168, 221, and 365. Prevaccination serum samples and serum samples harvested 221 and 365 days postvaccination were used for the studies of protective vaccine-induced antibodies presented here. All serum samples were heat inactivated at 56°C for 40 min before being used in experiments.

LAM-specific antibodies. LAM-specific IgG levels were measured by standard sandwich enzyme-linked immunosorbent assay (ELISA), as described elsewhere (3). All serum samples were studied at a dilution of 1:50.

Preparation of cells. Blood was obtained from healthy volunteers who were either PPD negative or positive. Neutrophils and peripheral blood mononuclear cells (PBMCs) were purified over density gradients (1-Step Polymorph [Accurate Chemical] and Histopaque [Sigma Diagnostics], respectively) according to the manufacturers' instructions. Monocytes/macrophages were isolated from PBMCs by plastic adherence as described previously (26). Dendritic cells were obtained from leukapheresed monocytes after they were stimulated with granulocyte-macrophage colony-stimulating factor and interleukin-4 (IL-4) for 6 days, followed by maturation induced by tumor necrosis factor alpha (TNF-α), IL-6, prostaglandin E2, and IL-1β for 24 h, as described previously (23). In some experiments, priming of neutrophils was achieved by incubating the cells with IFN-γ (1000 U/ml) and/or TNF-α (250 U/ml) for 30 min. All cells were resuspended in RPMI medium supplemented with 10% heat-inactivated human or fetal calf serum and 1% L-glutamine for the different experiments.

Surface binding and internalization of gfp-expressing BCG by neutrophils and monocytes/macrophages. BCG expressing green fluorescent protein (gfp-BCG) was provided by Michael O'Donnell, University of Iowa. gfp-BCG were pre-incubated with pre- or postvaccination sera diluted 1:2 in RPMI for 2 h at 37°C. The pretreated gfp-BCG were added to neutrophils (200,000/tube) or macrophages (50,000/tube) at a multiplicity of infection (MOI) of 3:1 for 2 h at 37°C. Thereafter, surface-bound BCG were stained with mouse monoclonal anti-LAM IgG_3 (Colorado State University, TB Research Unit) and goat anti-mouse antibody labeled with Alexa Fluor 647 (Molecular Probes). The cells were fixed with 1% formaldehyde and analyzed by flow cytometry. Cells positive for gfp but negative for Alexa Fluor 647 were considered to have only internalized BCG.

Growth inhibition of BCG. The general experimental design used for these experiments is summarized in Fig. 1A. Connaught strain BCG and 1:2 dilutions of pre- or postvaccination serum samples in RPMI were preincubated for 2 h at 37°C. Then neutrophils (150,000/well), monocytes/macrophages (15,000/well), PBMCs (150,000/well), or dendritic cells (15,000/well) were added to the BCG that had been preincubated with sera. A MOI of 1:1 was used for all experiments.

After 1 to 7 days, the viability of the BCG was assessed by culture and/or by the ^{3}H-uridine incorporation method. For each culture, aliquots were plated in triplicate on Middlebrook 7H10 agar and incubated for 3 weeks at 37°C before the numbers of CFU were counted. For measurement of the viability of the BCG by the ^{3}H-uridine uptake method, Saponin (0.2%) was added to lyse the mammalian cells and to release viable intracellular mycobacteria into the culture supernatant. One μCi of ^{3}H-uridine in 100 μl of Middlebrook 7H9 was added to each well containing 100 μl of saponin lysate. After 72 h at 37°C, the wells were harvested with an automated cell harvester (Harvester 96; Tomtec), using filter mats with a pore size of 0.45 μm. The radioactivity retained on the filters was measured in a beta counter (Microbeta; Trilux). To confirm that the observed effect on BCG growth was antibody mediated, we repeated some of the experiments with postvaccination sera after the immunoglobulins were depleted with Protein G (Sigma-Aldrich). The same experiments were also done without any phagocytic cells to exclude a cell-independent mycobactericidal effect of the postvaccination sera.

T-cell stimulation. The general design for these experiments is shown in Fig. 1B. For these T-cell expansion experiments, the Danish strain of BCG was used, as this type of assay had previously been optimized in our lab with this strain. BCG were preincubated with serum samples as described above. The pretreated BCG were then added to dendritic cells (3×10^5 to 5×10^5/tube) at a MOI of 2:1 and incubated for 2 h at 37°C. Meanwhile, nonadherent PBMCs were stained with 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) for 2 h at 37°C. The nonadherent PBMCs (10^6 cells) were then added to the dendritic cells in RPMI media supplemented with 10% heat-inactivated human serum, 1% L-glutamine, and 1% penicillin/streptomycin. After 7 days of incubation at 37°C, the expanded cells were restimulated with phorbol myristate acetate (50 ng/ml) and ionomycin (750 ng/ml) in the presence of monensin (GolgiStop; 0.7 μl/ml; BD Pharmingen) for 2 h. During this 2-h restimulation, anti-CD107a antibodies were added. Thereafter, the cells were stained for surface expression of CD4 and CD8, permeabilized for staining to detect intracellular expression of IFN-γ, and studied by flow cytometry.

Statistical analysis. Statistical analyses were performed with the software package Statistica 6.1 (StatSoft, Inc.). Experiments done with paired samples of pre- and postvaccination sera were analyzed by the Wilcoxon matched-pairs test. Repeated-measures analysis of variance (ANOVA) with Tukey post hoc comparisons were used to analyze differences in LAM-specific antibodies at different time intervals.

RESULTS

Intradermal BCG booster vaccination induces significant increases in mycobacterium-specific antibody responses. We had previously shown that BCG vaccination induces both serum and mucosal antibody responses directed against LAM (3, 11). In a new BCG vaccine trial, volunteers received two intradermal BCG vaccinations, given 6 months apart (12). We reasoned that individuals vaccinated twice with intradermal BCG would contain high levels of LAM-specific IgG and therefore would be good sources of serum samples for studies of the protective effects of mycobacterium-specific serum IgG. Shown in Fig. 2 are LAM-specific IgG ELISA results for serum samples harvested at different time intervals from the eight volunteers who received both doses of BCG, on day 0 and at 6 months. All volunteers were shown to develop increases in LAM-specific antibodies after the primary vaccination (Fig. 2A). The mean ELISA optical density levels shown in Fig. 2B were 0.576 at prevaccination, compared with 0.916 and 0.981 on days 56 and 112 postvaccination, respectively ($P < 0.05$ by repeated-measures ANOVA with post hoc Tukey HSD comparison). After the second BCG vaccination, the volunteers were found to have further increases in LAM-specific IgG antibodies. Mean optical density values in the LAM-specific IgG ELISA were 1.421 and 1.271 on days 221 and 365, respectively. Based upon these results, we chose the day 221 and 365 postvaccination serum samples for all further experiments designed to investigate the potential protective effects of mycobacterium-specific antibodies. To have a larger sample size, we included in subsequent experiments sera from the two volunteers who had received only one BCG vaccination in addition to the sera from the eight volunteers vaccinated twice. The two volunteers vaccinated only once had increased LAM-specific IgG antibodies at day 365, although the mean optical density for their sera appeared lower than the mean for the sera from the volunteers who had received two vaccinations (optical density, 0.681 versus 1.271).

Mycobacterium-specific antibodies enhance the internalization of BCG by neutrophils and monocytes/macrophages. Surface binding and internalization of BCG by neutrophils and monocytes/macrophages were studied, using gfp-expressing BCG and staining the surface-bound BCG with LAM-specific antibodies. These experiments showed that after pretreatment with postvaccination sera, the proportion of BCG associated with neutrophils after 2 h of incubation increased from a median of 75% to 90% ($P = 0.013$) (Fig. 3A). Furthermore, the proportion of gfp-positive neutrophils staining negatively for surface-bound BCG (Alexa Fluor 647$^-$) was 65% with prevaccination sera and 82% with postvaccination sera ($P = 0.013$) (Fig. 3B). These results indicate that neutrophils were more readily able to internalize BCG in the presence of antimycobacterial antibodies. There was no difference detected in the total number of monocytes/macrophages associated with gfp-BCG pretreated with pre- and postvaccination sera (Fig. 3C). However, with postvaccination sera, the internalization of BCG by monocytes/macrophages was increased, as previously observed with neutrophils. The proportion of monocytes/macrophages that had internalized BCG after 2 h of incubation increased from a median of 23% with prevaccination sera to a median of 32% with postvaccination sera ($P = 0.028$) (Fig. 3D).

Mycobacterium-specific antibodies enhance the phagocyte-mediated inhibition of BCG growth. To determine whether BCG-specific antibodies could enhance the killing of mycobacteria by phagocytic cells, we investigated the effects of pretreating BCG with paired samples of pre- and postvaccination sera before infecting purified neutrophils, monocytes/macrophages, total PBMCs, and blood-derived dendritic cells. As shown in Fig. 1A and described in Materials and Methods, serum-treated BCG were incubated with different cell preparations for 1 to 7 days before residual levels of viable BCG were quantified both by measuring incorporation of ^{3}H-uridine into mycobacterial RNA and by CFU plating.

In five independent experiments using different neutrophil preparations and all 10 pre- and postvaccination serum pairs, the mean level of ^{3}H-uridine incorporation by BCG after 24 h in the neutrophil/BCG coculture was 24% (standard error, ±3.6%) lower with postvaccination serum samples than with prevaccination serum samples. In an experiment with an incubation period of 72 h, postvaccination serum samples enhanced growth inhibition of BCG by neutrophils by a median of 25% as measured by ^{3}H-uridine incorporation and by a median of 33% as measured by CFU determination. Figure 4 shows the ^{3}H-uridine incorporation (panel A) and CFU (panel B) results from this experiment involving a neutrophil/BCG coculture period of 72 h.

Postvaccination serum samples containing mycobacterium-specific antibodies also enhanced the inhibitory effects of

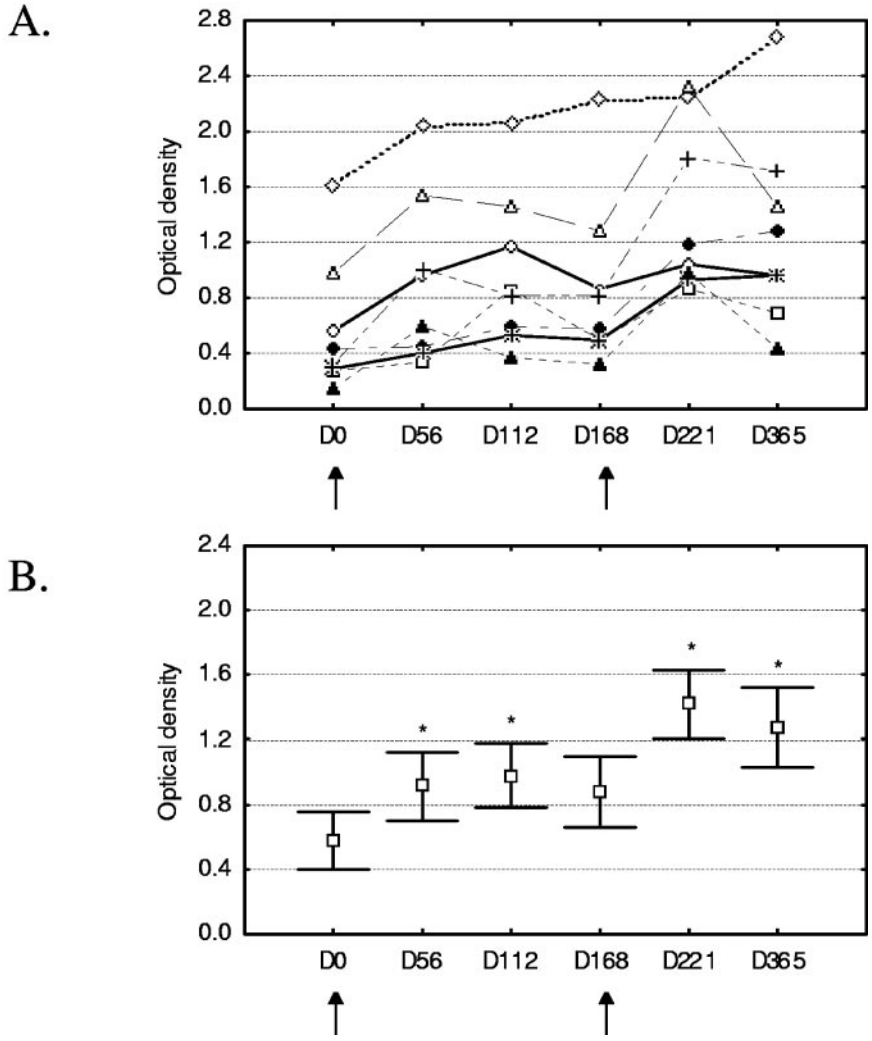

FIG. 2. Levels of LAM-specific IgG induced by primary and secondary intradermal BCG vaccinations. Each vaccination led to a significant increase in LAM-specific antibodies. Panel A shows the individual values for the eight volunteers who received two vaccinations, the first one on day 0 and the second one on day 168. The values for two additional volunteers were excluded because they did not receive the second vaccination on day 168. Panel B shows the mean values of the responses (small squares) and standard errors (bars). *, $P < 0.05$ by the Tukey HSD test with repeated-measures ANOVA. Arrows indicate the timing of the primary and secondary BCG vaccinations. D0, D56, D112, etc., represent days postvaccination. All serum samples were studied at a serum dilution of 1:50.

monocytes/macrophages on BCG growth. In three independent experiments, the mean ^{3}H-uridine uptake by BCG after 24 h of incubation was 26% (standard error, ±6.6%) lower in the presence of postvaccination serum samples than with prevaccination serum samples. After 72 h, the growth of BCG in BCG/monocyte/macrophage cocultures was decreased by a median of 34% as measured by ^{3}H-uridine incorporation (Fig. 4C) and by a median of 31% as measured by CFU plating (Fig. 4D) in the presence of postvaccination, compared with prevaccination, serum samples.

Similar experiments done with total PBMCs also demonstrated protective effects of mycobacterium-specific antibodies. After PBMCs, BCG, and paired serum samples were incubated for 24 h, the ^{3}H-uridine uptake was decreased by a median of 8% with postvaccination, compared with prevaccination, serum samples ($P = 0.036$).

In contrast to the results reported above for cultures of neutrophils, monocytes/macrophages, and total PBMCs, postvaccination serum samples did not enhance the inhibitory effects of dendritic cells on mycobacterial growth (data not shown).

Priming of neutrophils with different cytokines has been reported to increase the Fc receptor expression of neutrophils (15). We therefore were interested in the possibility that priming neutrophils with appropriate cytokines would further enhance the inhibitory effects of antimycobacterial antibodies. The overall inhibitory effects of neutrophils were enhanced by a median of 26% by IFN-γ priming and by a median of 35% by TNF-α priming. However, IFN-γ and/or TNF-α did not increase the growth-inhibiting effects of antimycobacterial antibodies observed with resting neutrophils (data not shown).

To demonstrate that the observed inhibitory effects on BCG growth were antibody-mediated, we studied postvaccination serum samples depleted of IgG with Protein G. This treatment reversed the growth-inhibiting effects of postvaccination serum

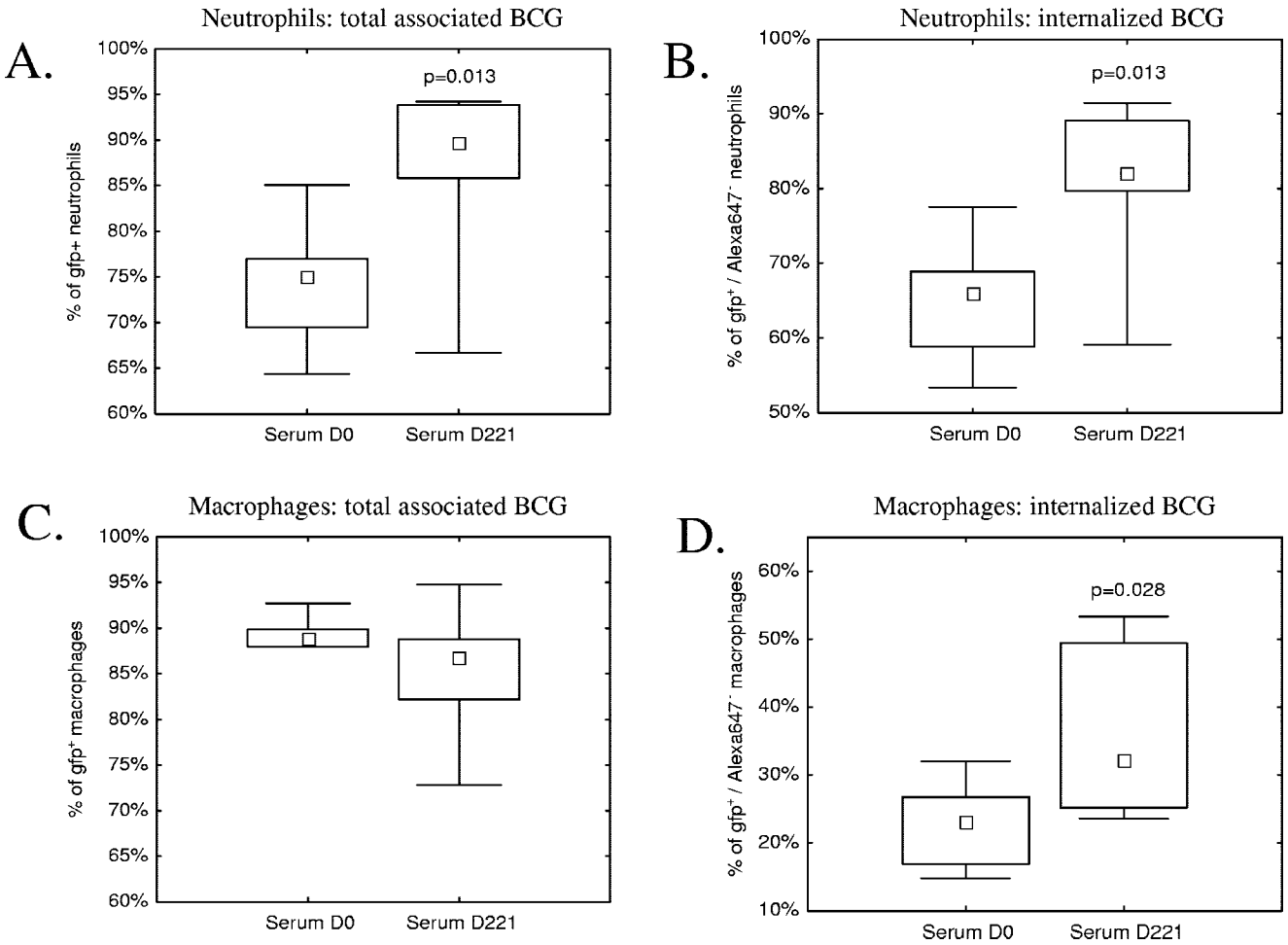

FIG. 3. Effect of postvaccination serum on surface binding and internalization of BCG by neutrophils and monocytes/macrophages. gfp-expressing BCG were pretreated with pre- or postvaccination serum and incubated with neutrophils or monocytes/macrophages. Two hours later, cells were fixed and surface-attached BCG were stained with anti-LAM mouse antibody and goat anti-mouse-IgG antibody labeled with Alexa Fluor 647. Shown are the overall uptake of gfp-expressing BCG by neutrophils (A) and monocytes/macrophages (C), as well as the proportions of neutrophils (B) and monocytes/macrophages (D) that were gfp^+/Alexa Fluor 647^-, indicating that they had internalized gfp-expressing BCG. Shown are medians (small squares), 25% to 75% ranges (boxes), and nonoutlier ranges (bars). *P* values are the results of Wilcoxon matched-pairs tests comparing results obtained with pre- and postvaccination serum samples. D0 and D221, days postvaccination.

samples seen in conjunction with phagocytic cells (data not shown).

Furthermore, we investigated whether mycobacterium-specific antibodies exerted cell-independent inhibitory effects on mycobacteria. BCG grew identically in the presence of pre- and postvaccination heat-inactivated serum samples without added cells (data not shown).

Mycobacterium-specific antibodies enhance the stimulation of cell-mediated immune responses. Antigens coated with specific antibodies can be internalized via the Fc receptor pathway. This has been shown to enhance the ability of antigen-presenting cells to stimulate T-cell responses directed against a number of pathogens (14). Therefore, we investigated whether antibodies induced by BCG vaccination could enhance mycobacterium-specific $CD4^+$ and $CD8^+$ T-cell responses. The general design of these experiments is shown schematically in Fig. 1B and described in more detail in Materials and Methods. Briefly, T cells were labeled with CFSE, which can be used to identify antigen-specific T cells that proliferate in response to stimulation because they reduce in half their green fluorescence intensity with each cell division. Aliquots of BCG pretreated with pre- and postvaccination serum samples were used to infect dendritic cells. These infected dendritic cells were used as antigen-presenting cells to stimulate CFSE-labeled T cells. After 7 days, expanded T cells were studied after surface and intracellular staining followed by flow cytometry. After gating on $CD4^+$ or $CD8^+$ lymphocytes, the percentages and absolute numbers of antigen-specific, $CFSE^{lo}$, IFN-γ^+, and $CD107a^+$ cells were identified. $CD107a^+$ staining detects transient surface expression of lysome-associated membrane protein 1 during degranulation.

Figure 5 demonstrates that pretreating BCG with postvaccination serum before infecting dendritic cells significantly increased the percentage of responding $CD4^+$, $CFSE^{lo}$, and IFN-γ^+ cells. Panels A and B present representative dot plots for experiments conducted with one pair of pre- and postvac-

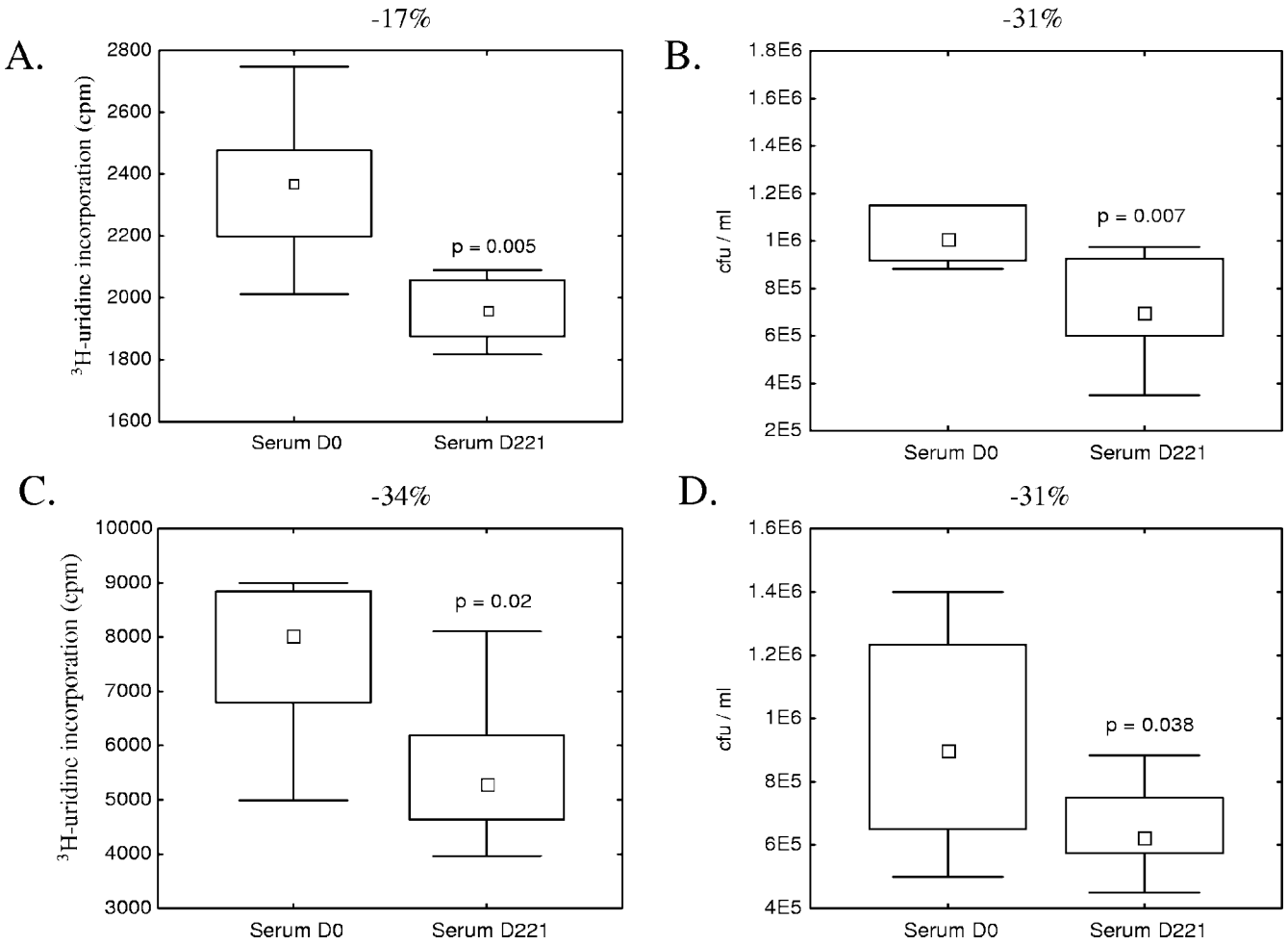

FIG. 4. Enhancement of the inhibitory effects of phagocytic cells on BCG growth by mycobacterium-specific antibodies. BCG were pretreated with paired samples of pre- and postvaccination sera and then incubated with purified neutrophils or monocytes/macrophages for 72 h. Shown are the persistent levels of viable BCG as measured by ^{3}H-uridine uptake (A and C) and by CFU determination (B and D). These results demonstrate that growth inhibition is enhanced by preincubating BCG with postvaccination serum. Shown are medians (small squares), 25% to 75% ranges (boxes), and nonoutlier ranges (bars). *P* values are the results of Wilcoxon matched-pairs tests comparing results obtained with pre- and postvaccination serum samples. The percentages above each graph represent the median reduction in BCG growth after pretreatment with postvaccination versus prevaccination sera. D0 and D221, days postvaccination.

cination serum samples. Panel C shows the medians and ranges of responses for an experiment conducted with the paired serum samples from all 10 volunteers. The median percentage of $CD4^+$, $CFSE^{lo}$, and IFN-γ^+ cells with prevaccination serum was 27.1% compared with 32.7% with postvaccination serum ($P = 0.02$). In a second experiment determining absolute cell numbers, the median number of $CD4^+$, $CFSE^{lo}$, and IFN-γ^+ T cells increased from 1.5×10^5 cells/ml with prevaccination serum to 2.1×10^5 cells/ml with postvaccination serum ($P = 0.09$).

Postvaccination serum samples containing mycobacterium-specific antibodies were shown to have even greater enhancing effects on the capacity of dendritic cells to stimulate BCG-specific $CD8^+$ T cells. The data presented in Fig. 6 demonstrate that pretreatment of BCG with postvaccination serum samples resulted in significantly increased expansion of antigen-specific $CD8^+$ T cells capable of producing IFN-γ. The median percentage of $CD8^+$, $CFSE^{lo}$, and IFN-γ^+ T cells increased from 14.8% with prevaccination serum to 24.1% with postvaccination serum ($P = 0.007$). In the second experiment determining absolute cell numbers, the median number of $CD8^+$, $CFSE^{lo}$, and IFN-γ^+ T cells increased from 1.0×10^5 cells/ml with prevaccination serum to 1.8×10^5 cells/ml with postvaccination serum ($P = 0.05$).

Furthermore, the data presented in Fig. 7 confirms that these enhanced $CD8^+$ T cells were capable of cytolytic degranulation. The median percentage of $CD8^+$, $CFSE^{lo}$, and $CD107a^+$ T cells increased from 6.3% with prevaccination serum to 8.5% with postvaccination serum ($P = 0.007$). In the second experiment determining absolute cell numbers, the median number of $CD8^+$, $CFSE^{lo}$, and $CD107a^+$ T cells increased from 0.7×10^5 cells/ml with prevaccination serum to 1.1×10^5 cells/ml with postvaccination serum ($P = 0.03$).

Similar results were seen when T cells from either PPD-positive or PPD-negative individuals were used for these experiments. A summary of the results of four independent ex-

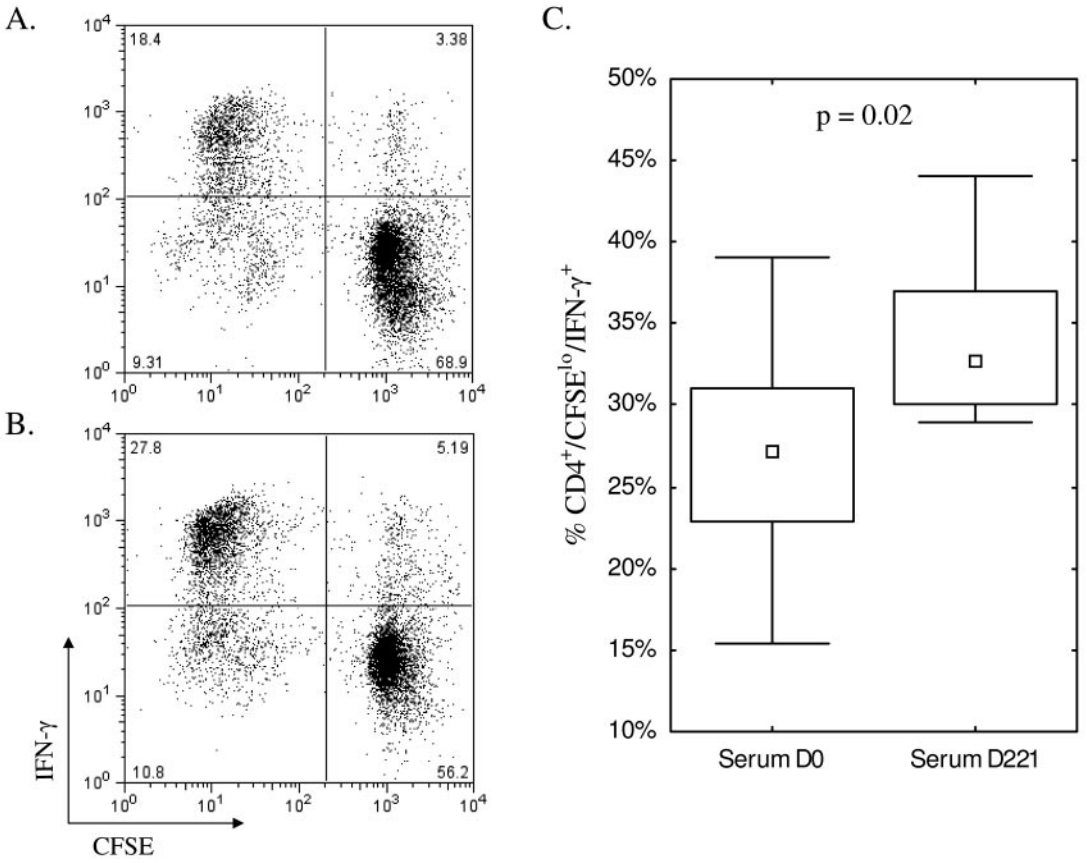

FIG. 5. Enhancement of $CD4^+$ T-cell responses by mycobacterium-specific antibodies. T-cell expansion in the presence of BCG, dendritic cells, and postvaccination sera showed an increased proportion of proliferating (CFSE-low) and IFN-γ expressing $CD4^+$ T cells. Shown are representative results of four independent experiments with all 10 pre- and postvaccination serum pairs. Presented in panels A and B are representative fluorescence-activated cell sorter (FACS) plots demonstrating effects on $CD4^+$ T cells of paired pre- (A) and postvaccination (B) sera from one volunteer. Panel C shows a summary of effects of pre- and postvaccination sera from one experiment with all 10 volunteers. $P = 0.02$ by the Wilcoxon matched-pairs test. D0 and D221, days postvaccination. Medians (small squares), 25 to 50% ranges (boxes), and nonoutlier ranges (bars) are shown.

periments done with cells from PPD-positive and PPD-negative individuals is shown in Table 1.

DISCUSSION

Current concepts about protective immune mechanisms active against TB emphasize the almost exclusive role of cell-mediated immunity. However, the data presented here indicate that antimycobacterial antibodies have important immune effects, which may play a significant role in various stages of the host response to TB infection. Neutrophils and monocytes/macrophages showed increased internalization and increased killing of mycobacteria in the presence of specific antibodies. In addition, mycobacteria coated with specific antibodies were more effectively processed and presented by dendritic cells for stimulation of $CD4^+$ and $CD8^+$ T-cell responses. These findings should stimulate a reassessment of the importance of antibody responses for TB vaccine development.

The specific mycobacterial targets for antibodies mediating the enhanced internalization and inhibition of mycobacteria by phagocytic cells, as well as the increased T-cell stimulation, are not known. Confirming earlier work (3), vaccination with BCG again led to a significant increase in LAM-specific antibodies (Fig. 2). Both the primary vaccination and the booster vaccination induced increases of LAM-specific antibodies in all volunteers. The booster vaccination induced a larger increase in LAM-specific antibodies, although this difference was not statistically significant. The prevaccination levels of LAM-specific reactivity in serum showed considerable variation, suggesting the presence of pre-existing LAM-specific antibodies, although all volunteers were PPD-negative and had no known exposure to tuberculosis. It is likely that these antibodies had been induced through prior exposure to environmental mycobacteria or other cross-reactive environmental antigens. The baseline presence of mycobacterium-specific antibodies, however, did not prevent the induction of LAM-specific antibody responses by the two BCG vaccinations. Further studies are indicated to determine whether the inhibitory effects on mycobacteria and/or the enhancing effects on T-cell activation detected here are due to LAM-specific antibodies.

Both neutrophils and monocytes/macrophages are able to ingest mycobacteria. However, it is believed that mycobacteria are able to resist intracellular killing by inhibition of phagosome-lysosome fusion and suppression of the respiratory burst

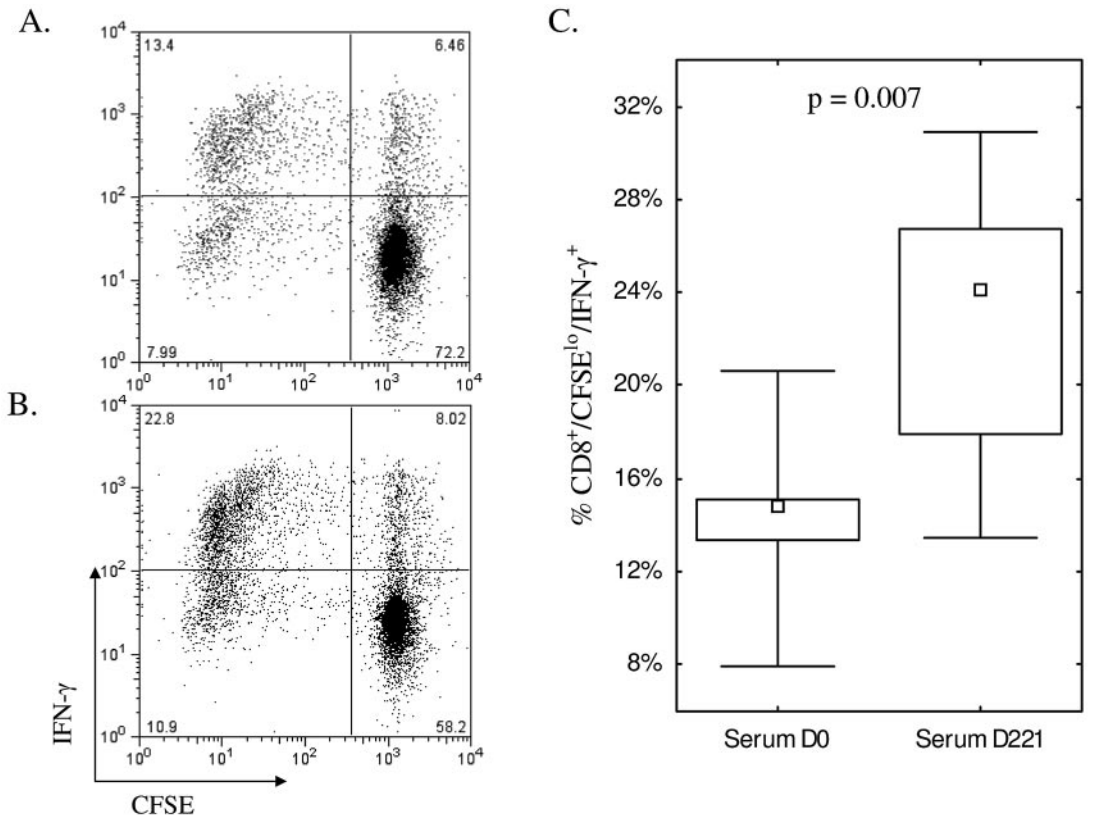

FIG. 6. Enhancement of $CD8^+$ T-cell responses by mycobacterium-specific antibodies. T-cell expansion in the presence of BCG, dendritic cells, and postvaccination sera showed an increased proportion of proliferating (CFSE-low) and IFN-γ-expressing $CD8^+$ T cells. Shown are representative results of four independent experiments with all 10 pre- and postvaccination serum pairs. Presented in panels A and B are representative FACS plots demonstrating effects on $CD8^+$ T cells of paired pre- (A) and postvaccination (B) sera from one volunteer. Panel C shows a summary of effects of pre- and postvaccination sera from one experiment with all 10 volunteers. $P = 0.007$ by the Wilcoxon matched-pairs test. D0 and D221, days postvaccination. Medians (small squares), 25 to 50% ranges (boxes), and nonoutlier ranges (bars) are shown.

(9). Phagocytic cells engulf mycobacteria via different surface receptors, normally involving complement receptors and mannose-binding receptors (16, 18). The opsonization of mycobacteria with antibodies increased the internalization and killing of BCG by phagocytic cells, and we believe that these effects were most likely mediated by Fc receptors. Uptake via complement or mannose binding receptors compared with that via Fc receptors may lead to very different consequences for viable mycobacteria. For example, uptake via CR3 can be advantageous for mycobacterial survival (17). On the other hand, uptake via Fc receptors has been shown to activate intracellular microbicidal activities against several pathogens (4). In addition, earlier studies demonstrated that the ability of *M. tuberculosis* to inhibit phagosome-lysosome fusion was reversed by first precoating the pathogen with specific immune serum (1, 13). Therefore, it is possible that the mechanism of enhanced growth inhibition associated with mycobacterium-specific antibodies identified in the present work is related to a differential targeting of mycobacteria for uptake via Fc receptors rather than complement or mannose binding receptors, resulting in enhanced phago-lysosome formation and intracellular killing of mycobacteria.

Enhancement of the antimycobacterial activity of phagocytic cells by BCG-induced antibodies could be particularly important in the context of mucosal immunity. Antibodies of the IgG and IgA classes have been shown to be present in the mucosal secretions of the lower respiratory tract (2). Alveolar macrophages and neutrophils in the respiratory mucosa are among the first immune cells encountered by invading mycobacteria. Enhancement of the antimycobacterial functions of these cells is likely to be important for limitation of early mycobacterial proliferation and dissemination before cell-mediated immunity develops. It has been shown that pulmonary neutrophilia induced by intratracheal injection of lipopolysaccharides dramatically decreased the number of mycobacteria that can subsequently be recovered from rat lungs after aerosol challenge with *M. tuberculosis* (20). Both neutrophils and macrophages are also sources of cytokines, which are important in the chemoattraction of other immune cells as well as in granuloma formation (19, 27). Depending on the type of stimuli, neutrophils and macrophages have been shown to produce different profiles of cytokines. For example, the uptake by neutrophils of *Saccharomyces cerevisiae* opsonized with IgG led to an increased secretion of the cytokines IL-8 and TNF-α (5). Modulation of the cytokine profile induced by antibody-coated mycobacteria is possible and warrants further investigation.

Mycobacterium-specific antibodies also had enhancing effects on cell-mediated immunity. In the presence of dendritic

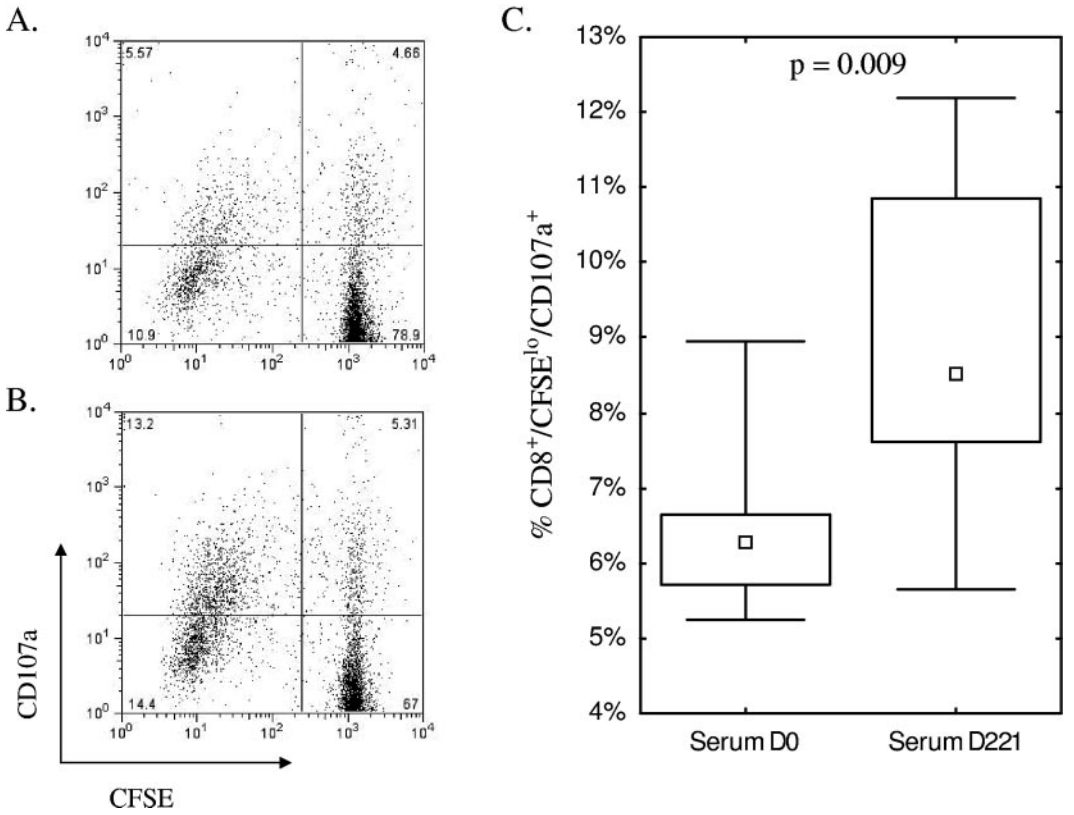

FIG. 7. Enhancement of $CD8^+$ T-cell responses by mycobacterium-specific antibodies. T-cell expansion in the presence of BCG, dendritic cells, and postvaccination sera showed an increased proportion of proliferating (CFSE-low) and degranulating (CD107a-high) $CD8^+$ T cells. Shown are representative results of four independent experiments with all 10 pre- and postvaccination serum pairs. Presented in panels A and B are representative FACS plots demonstrating effects on $CD8^+$ T cells of paired pre- (A) and postvaccination (B) sera from one volunteer. Panel C shows a summary of effects of pre- and postvaccination sera from one experiment with all 10 volunteers. $P = 0.009$ by the Wilcoxon matched-pairs test. D0 and D221, days postvaccination. Medians (small squares), 25 to 75% ranges (boxes), and nonoutlier ranges (bars) are shown.

cells as antigen-presenting cells, antimycobacterial antibodies led to significant increases in the proportions and absolute numbers of proliferating and IFN-γ-expressing $CD4^+$ and $CD8^+$ T cells after stimulation with BCG. The expression of the surface molecule CD107a, which has been shown to be a marker of degranulation of lymphocytes, was also increased on the $CD8^+$ T cells stimulated by BCG in the presence of mycobacterium-specific antibodies.

The increased stimulation of T cells obtained with mycobacterium-specific antibodies depended on the degree of baseline stimulation observed with the use of prevaccination serum. The largest effects of antibodies were observed in T cells harvested from individuals displaying a low baseline response, while the weakest effects were observed with T cells from individuals already showing a strong baseline response without the presence of mycobacterium-specific antibodies. These results could be important for the design of future vaccines. If a stronger cell-mediated immune response can be obtained after initial induction of antimycobacterial antibodies, it might be worthwhile to consider a priming-boosting strategy which initially induces a strong humoral response and then focuses on T-cell stimulation. An alternative would be to vaccinate with BCG that has been passively coated with antibodies to elicit a stronger cell-mediated immune response. Consistent with this idea, antibody-coated chlamydiae were previously shown to induce better protective T-cell immunity than chlamydiae administered without specific antibody coating (14).

TABLE 1. Mycobacterium-specific antibodies enhance T-cell expansion and effector functions

Cell type	Increase due to antimycobacterial antibodies[a]
$CD4^+/CFSE^{lo}/IFN\text{-}\gamma^+$	19.7% ± 5.6%
$CD8^+/CFSE^{lo}/IFN\text{-}\gamma^+$	32.1% ± 14.1%
$CD8^+/CFSE^{lo}/CD107a^+$	23.1% ± 7.0%

[a] Values are mean relative changes ± standard errors of the different T-cell subpopulations from four independent T-cell expansion experiments.

Considering that about one-third of the world's population is latently infected with *M. tuberculosis* and that the lifetime risk of reactivation is about 10% in otherwise healthy individuals with latent infection, it would be useful to have a vaccine that can induce an immune response protective against reactivation. IFN-γ and $CD8^+$ T cells have been demonstrated to be important for control of the latent stage of *M. tuberculosis* (21, 24). As mycobacterium-specific antibodies enhance activation of both IFN-γ-producing T cells and degranulating $CD8^+$ cells, their induction and possibly regular boosting might reduce the risk of TB reactivation.

In summary we have shown that vaccination with BCG induces significant increases in LAM-specific IgG and that BCG-induced antibodies promote important enhancing effects on both the innate and cell-mediated immune responses to mycobacteria. Future studies will address the specific mechanisms involved in antibody-mediated immune enhancement and whether these effects can be utilized to develop more-potent TB vaccines.

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