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Priming-Boosting Vaccination with Recombinant *Mycobacterium bovis* Bacillus Calmette-Guérin and a Nonreplicating Vaccinia Virus Recombinant Leads to Long-Lasting and Effective Immunity

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Virus-specific T-cell responses can limit immunodeficiency virus type 1 (HIV-1) transmission and prevent disease progression and so could serve as the basis for an affordable, safe, and effective vaccine in humans. To assess their potential for a vaccine, we used *Mycobacterium bovis* bacillus Calmette-Guérin (BCG)-Tokyo and a replication-deficient vaccinia virus strain (DIs) as vectors to express full-length *gag* from simian immunodeficiency viruses (SIVs) (rBCG-SIVgag and rDIsSIVgag). Cynomolgus macaques were vaccinated with either rBCG-SIVgag dermally as a single modality or in combination with rDIsSIVgag intravenously. When cynomologus macaques were primed with rBCG-SIVgag and then boosted with rDIsSIVgag, high levels of gamma interferon (IFN-γ) spot-forming cells specific for SIV Gag were induced. This combination regimen elicited effective protective immunity against mucosal challenge with pathogenic simian-human immunodeficiency virus for the 1 year the macaques were under observation. Antigen-specific intracellular IFN-γ activity was similarly induced in each of the macaques with the priming-boosting regimen. Other groups receiving the opposite combination or the single-modality vaccines were not effectively protected. These results suggest that a recombinant *M. bovis* BCG-based vector may have potential as an HIV/AIDS vaccine when administered in combination with a replication-deficient vaccinia virus DIs vector in a priming-boosting strategy.

As the rate of new infections with human immunodeficiency virus type 1 (HIV-1) continues to increase globally, an effective preventive vaccine is urgently needed to stem further spread of the virus (24). Because long-term survival in humans has been observed when HIV-1 replication is controlled by protective immunity (12, 29), targeted experimental immunogens have been designed to closely mimic the long-lasting protective immunity induced in long-term human survivors by the natural infection (8, 25). Recently, various vaccine modalities, including live viral vectors and DNA, have been used to elicit protective immunity in nonhuman primate models (9). However, before an HIV-1 vaccine regimen can be considered promising, it must be shown to be not only effective at inducing protective immunity, but also safe, affordable, and compatible with other vaccines (2, 32).

When it comes to safety, traditional live vaccines, which have been administered safely to both the healthy and the infected, may be the vectors of choice for HIV-1 vaccines. In order to fully take advantage of the potential benefits of traditional live vectors in HIV-1 vaccine development, we studied the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) substrain Tokyo 172 (6) and the replication-deficient vaccinia virus vaccine strain DIs (22, 50), both of which have been shown to be nonpathogenic when inoculated into immunodeficient animals (41, 51, 53) as live recombinant vaccine vehicles (1, 17–19, 46–48). As further evidence of the potential of the live vectors for use in HIV/AIDS vaccines, we noted that a recombinant *M. bovis* BCG vector candidate vaccine for HIV-1-induced positive immune responses in animals (17, 46). Moreover, we found that recombinant vaccinia virus DIs encoding the simian immunodeficiency virus (SIV) gene was effective at eliciting anti-SIV immunity in mice when administered as a booster antigen after priming with SIV DNA (47). In this study, we have developed a new combination regimen, priming with recombinant *M. bovis* BCG-SIV Gag followed by boosting with rDIsSIVgag. This immunization regimen elicited effective positive immunity against an immune deficiency virus in macaques for the 1 year they were under study.

MATERIALS AND METHODS

Animals and virus challenge stocks. All animals used in this study were captive bred and obtained from the Philippines. They were mature, cycling, male cynomologus macaques (*Macaca fascicularis*) from the Tsukuba Primate Center, the National Institute of Infectious Diseases, Japan. Animals used in these studies were free of known simian retroviruses, herpes viruses, bacteria, and parasites. They were housed in accordance with the Guidelines for Animal Experimenta-

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tion of the Japanese Association for Laboratory Animal Science, 1987, under the Japanese Law Concerning the Protection and Management of Animals (46) and were maintained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of National Institute of Infectious Diseases, Japan. Once approved by an institutional committee for biosafety level 3 experiments, these studies were conducted at the Tsukuba Primate Center, National Institute of Infectious Diseases, Japan, in accordance with the requirements specifically stated in the laboratory biosafety manual of the World Health Organization. The animals' condition was monitored by analyzing a hemogram parameter as well as absolute $CD4^+$ and $CD8^+$ T-lymphocyte counts with an automated blood analyzer Celltac (Nihon Koden, Tokyo Japan), as described below.

Two thousand 50% tissue culture infectious doses ($TCID_{50}$) of SHIV KS661c, a pathogenic molecular clone, were intrarectally administered as a challenge virus (39). The parent virus, SHIV-C2/1, is an SHIV-89.6 variant isolated by in vivo passage in cynomolgus macaques (40, 42) and the original SHIV-89.6 strain was kindly provided by Y. Lu at the Harvard AIDS Institute (Boston, MA) (26, 37). SHIV-C2/1 and SHIV KS661c were shown to infect cynomologus macaques by both the intravenous and intrarectal routes (39). Both viruses induced high levels of viremia and marked $CD4^+$ T-cell depletion within 2 and 3 weeks after inoculation, respectively (39, 40, 42). Virus stocks were stored at −125°C and thawed just prior to use.

Production and preparation of recombinant *M. bovis* BCG (rBCG) and vaccinia virus DIs expressing full-length SIV Gag. Detailed methods for plasmid construction were described previously (7, 17, 18, 21). Briefly, a DNA fragment encoding the full-length *gag* sequence of SIVmac239 was cloned downstream of the *hsp60* promoter (52) and then inserted into the multicloning site of the plasmid pSO246 (28). Recombinant *Mycobacterium bovis* BCG substrain Tokyo 172 that stably expressed the inserted DNA fragment (designated rBCG-SIVgag) was then selected and used for all rBCG inoculations. For the Western blot analysis, the transformant of rBCG was grown in 7H9-ADC broth for 2 weeks and a portion of the culture medium was periodically collected, sonicated and blotted using the monoclonal antibody IB6, as described previously (47). Since the recombinant DIs virus (rDIs) encoding the SIVmac239 *gag-pol* open reading frame elicited remarkably high SIV Gag-specific T-cell responses but low polymerase responses in mice (47), confirming the findings of a previous report (20), we named it rDIsSIVgag. The rDIsSIVgag and rDIs encoding β-galactosidase (rDIsLacZ) were prepared with chicken embryo fibroblast (CEF) cells (18, 47). Virus preparations were purified by sucrose density gradient ultracentrifugation and were adjusted to 10^7 PFU/ml. P27 antigen generation in cells was measured by antigen-specific enzyme-linked immunosorbent assay (42).

Virus-specific IFN-γ ELISPOT assays. ELISPOT assays were performed using the method developed by and following the direct instructions of Mothe and Watkins, Wisconsin University Primate Center (31, 46). In brief, 96-well flat-bottomed plates (U-CyTech-BV, Utrecht, Netherlands) were coated with anti-gamma interferon (IFN-γ) monoclonal antibody MD-1 (U-CyTech-BV). Freshly isolated peripheral blood mononuclear cells (PBMC) were added with either concanavalin A or pooled Gag peptides (AIDS Research and Reference Reagent Program, National Institutes of Health, Rockville, MD). The cells were then incubated in anti-IFN-γ-coated plates before lysing with ice-cold deionized water. After plates had been washed, rabbit anti-IFN-γ polyclonal biotinylated detector antibody (1 μg per well; U-CyTech-BV) was added. The plates were reacted with gold-labeled antibiotin immunoglobulin G solution by adding 30 μl of the activator mix (U-CyTech-BV) to each of the wells and allowing them to develop for 15 min.

Wells were imaged and spot-forming cells (SFC) were counted using the KS ELISPOT compact system (Carl Zeiss, Germany) (31, 46). An SFC was defined as a large black spot with a fuzzy border. To determine significance levels, we established a baseline for each peptide using the average and standard deviation of the number of SFC for each peptide. A threshold significance value corresponding to this average and two standard deviations were then determined. A response was considered positive if the number of SFC exceeded the threshold significance level of the sample with no added peptide.

Detection of intracellular IFN-γ by flow cytometry. Intracellular macaque IFN-γ was detected by intracellular IFN-γ cytokine staining as previously described (30). Briefly, freshly isolated PBMC was incubated with antigen for 16 h at 37°C with 5% CO_2. During the final 6 to 8 h, brefeldin A (Sigma Chemical Co., St. Louis, MO) was added at 10 μg/ml. Antibody to CD28 (1 μg/ml, BD Pharmingen, San Diego, CA) was also added during the incubation as a costimulator molecule. After stimulation, the cells were stained with fluorescein isothiocyanate-conjugated anti-CD3 (FN18; Biosource, Camarillo, CA) and peridinin chlorophyll protein-conjugated anti-CD8 antibodies (Leu-2a; Becton Dickinson Biosciences, San Jose, CA). The cells were then sequentially incubated with fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson) for 10 min and FACS permeabilizing solution (Becton Dickinson) for another 10 min. The cells were washed, stained with phycoerythrin-conjugated anti-human IFN-γ antibody (4S.B3; BD Pharmingen), and fixed with 2% paraformaldehyde. Samples were analyzed with a FACSCalibur using Cell Quest software (Becton Dickinson).

Lymphocyte proliferative responses. SIV-specific proliferative responses were measured in freshly isolated PBMC as described by Gauduin et al. and Hel et al. (14, 15). PBMC were cultured in flat-bottomed 96-well plates with either concanavalin A or purified SIVmac251 p27 protein (Advanced BioScience Laboratories, Rockville, MD) (15) for three days before the addition of [^{3}H]thymidine. Cells were harvested 16 h later to determine uptake.

Absolute $CD4^+$ and $CD8^+$ T-lymphocyte counts. An absolute cell count of peripheral blood was measured as previously described (55). Briefly, 50 μl of whole blood was placed in a polypropylene tube and incubated with FITC-conjugated monoclonal anti-CD3 (FN18; Biosource), phycoerythrin-conjugated anti-CD4 (Leu-3a; Becton Dickinson), and peridinin chlorophyll protein-conjugated anti-CD8 (Leu-2a; Becton Dickinson) antibodies at 4°C. After incubation with FACS lysing solution (Becton Dickinson), the cells were analyzed with a FACSCalibur using Cell Quest software (Becton Dickinson).

Plasma viral RNA copy numbers. Plasma viral RNA copy numbers were measured using a real-time quantification assay based on the TaqMan system (Applied Biosystems, Foster City, CA) and the Prism 7700 sequence detection system (Applied Biosystems), as reported previously (30, 46). Briefly, viral RNA was extracted and purified from macaque plasma samples using a QIAamp viral RNA mini kit (QIAGEN, Valencia, CA). The RNA was subjected to reverse-transcription and amplification using a TaqMan EZ RT-PCR Kit (Applied Biosystems) with SIV Gag consensus primers SIVmac239-1224F and SIVmac239-1326R, and the SIV Gag consensus Taqman probe FAM-SIV-1272T. To obtain control RNA for quantification, SIVmac239 *gag* RNA was synthesized using T7 RNA polymerase and pKS460, a template plasmid that contains SIVmac239 *gag* under control of the T7 promoter.

To measure the RNA recovery rate, 10^5 copies of SHIV KS661c, in which the viral RNA copy number was previously determined by branched DNA assay (Bayer), were extracted and purified using the same kit as for the sample. Plasma viral load was calculated based on the standard curve of control RNA and the RNA recovery rate. All assays were carried out in duplicate.

Statistical analysis. Data analysis was carried out using the Stat View program (SAS Institute, Cary, NC) and data are expressed as the mean ± standard deviation. A P value of <0.05 was considered significant.

RESULTS

Construction and preparation of recombinant *M. bovis* BCG Tokyo 172 and vaccinia virus DIs expressing whole SIV Gag. In initial studies, we cloned DNA encoding SIVmac239 *gag* downstream of the *hsp60* promoter and the expression unit was inserted into the KpnI restriction site of plasmid pSO246. We also constructed a recombinant *M. bovis* BCG vaccine based on the Tokyo 172 strain expressing the full-length *gag* gene of SIVmac239 (rBCG-SIVgag) (Fig. 1A). The presence of SIV Gag-specific DNA was confirmed in recombinant bacteria by DNA-PCR (42). To determine the in vitro expression of the SIV Gag protein in the cells, we analyzed cell extracts of rBCG-SIVgag bacteria after 2 weeks of culture by Western blot using anti-SIV Gag monoclonal antibody IB6. The rBCG clone produced an SIV Gag recombinant protein that strongly reacted as a single band with the specific monoclonal antibody (Fig. 1B). The concentration of SIV Gag^{p27} protein in transformed bacteria was 28.56 ± 8.30 ng/10^8 CFU of bacilli. In contrast, neither SIV Gag protein nor *gag* DNA was detected in bacteria transformed with rBCG-pSO246, a control construct lacking the SIV *gag* insert and used as a vector control (Fig. 1B).

rDIsSIV*gag* and a control vaccinia virus, rDIsLacZ, were propagated in CEF and adjusted to 10^7 PFU/ml. Using Western blot, we confirmed the expression of each foreign gene in

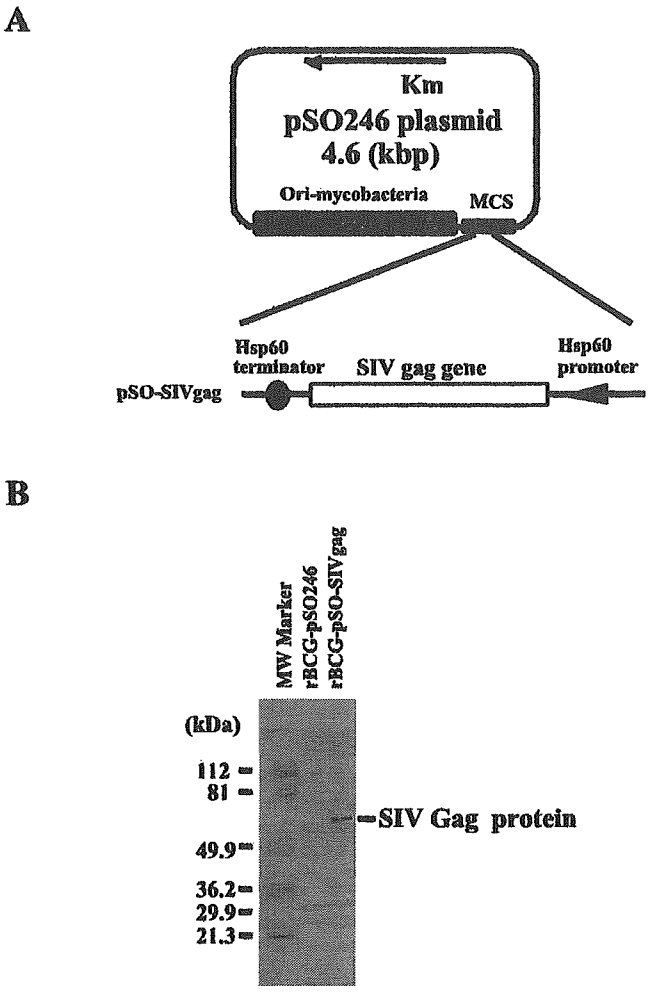

FIG. 1. Vector construction and expression of rBCG-SIVgag. (A) Construction of the expression vector pSO-SIVgag. Full-length DNA of SIVmac239 *gag* was inserted into the multicloning site of pSO246 and expressed in the vaccine strain *M. bovis* BCG Tokyo 172. (B) Detection of SIV Gag protein by Western blot with anti-p27 Gag monoclonal antibody IB6.

the cell extract, and purified virions used as immunogens in this study.

Immune induction after single-modality or combined immunization regimens with vaccine candidates. We examined whether rBCG expressing the full-length *gag* gene of SIVmac239 would be suitable for use in combined prime-boost protocols with the replication-deficient vaccinia virus strain DIs recombinant. The rBCG was intradermally delivered to the inner region of the thigh and rDIs was intravenously administered into the small saphenous vein on the back of the leg. Of the 15 macaques registered in this study, 13 were divided into five groups and immunized using either a single-modality regimen plus vector controls or with a priming-boosting regimen consisting of *M. bovis* BCG and vaccinia virus DIs recombinants (Table 1). The remaining two macaques were inoculated with phosphate-buffered saline and served as naïve controls throughout the experiment.

Group 1 (control group, $n = 3$) served as a vector control group that received rBCG-pSO246 intradermally followed by two inoculations of rDIsLacZ intravenously; group 2 (rBCG group, $n = 2$) received rBCG-SIVgag intradermally followed by two inoculations of rDIsLacZ intravenous, while group 3 (rBCG/rDIs group, $n = 3$) received rBCG-SIVgag intradermally followed by two inoculations of rDIsSIVgag intravenously. Finally, group 4 (rDIs group, $n = 2$) received two inoculations of rDIsSIVgag intravenously followed by rBCG-pSO246 intradermally, while group 5 (rDIs/rBCG group, $n = 3$) received two inoculations of rDIsSIVgag intravenously followed by rBCG-SIVgag intradermally. The 13 immunized and two naive animals were studied for immune induction for 64 weeks before being mucosally challenged with virulent SHIV for a period of 1 year (Table 1).

Antigen-specific T-cell responses in all 15 animals were monitored by SIV Gag peptide-specific IFN-γ-ELISPOT assays (Fig. 2). Fifty weeks postinfection, the rBCG/rDIs group showed the highest SIV Gag-specific IFN-γ-ELISPOT responses; that group's responses peaked at 1,020 ± 360 SFC/10^6 PBMC at 56 weeks postinfection or 2 weeks after the second booster inoculation (Fig. 2A). At 56 weeks postinfection, the ELISPOT responses of the rDIs/rBCG group (380 ± 35 spots per million PBMC, Fig. 2B) were significantly lower than those of the rBCG/rDIs group ($P < 0.05$), as were the ELISPOT

TABLE 1. Immunization and challenge schedule[a]

Group no. (regimen)	Macaque no.	Priming immunization, route, and schedule	Boost immunization, route, and schedule	Mucosal challenge[b]
1 (control)	06, 90, and 91	rBCG-pSO246, 10 mg, i.d., wk 0	rDIsLacZ, 10^6 PFU, i.v., wk 47 and 54	2,000 $TCID_{50}$, i.r., wk 64
2 (rBCG)	29 and 93	rBCG-SIV *gag*, 10 mg, i.d., wk 0	rDIsLacZ, 10^6 PFU, i.v., wk 47 and 54	2,000 $TCID_{50}$, i.r., wk 64
3 (rBCG/rDIs)	08, 10, and 46	rBCG-SIVgag, 10 mg, i.d., wk 0	rDIsSIVgag, 10^6 PFU, i.v., wk 47 and 54	2,000 $TCID_{50}$, i.r., wk 64
4 (rDIs)	01 and 42	rDIsSIVgag, 10^6 PFU, i.v., wk 0 and 8	rBCG-pSO246, 10 mg, i.d., wk 54	2,000 $TCID_{50}$, i.r., wk 64
5 (rDIs/rBCG)	85, 36, and 40	rDIsSIVgag, 10^6 PFU, i.v., wk 0 and 8	rBCG-SIVgag, 10 mg, i.d., wk 54	2,000 $TCID_{50}$, i.r., wk 64

[a] Vaccines, immunization, and challenge studies for all the macaques are described in the text. Animal studies were simultaneously conducted using cynomolgus macaques. i.d., intradermal inoculation; i.v., intravenous inoculation; i.r., intrarectal inoculation.

[b] All of the animals were mucosally challenged with virulent SHIV KS661c at 64 weeks postimmunization and were observed for at least 1 year or, if they did not survive for a year, until the time of their death.

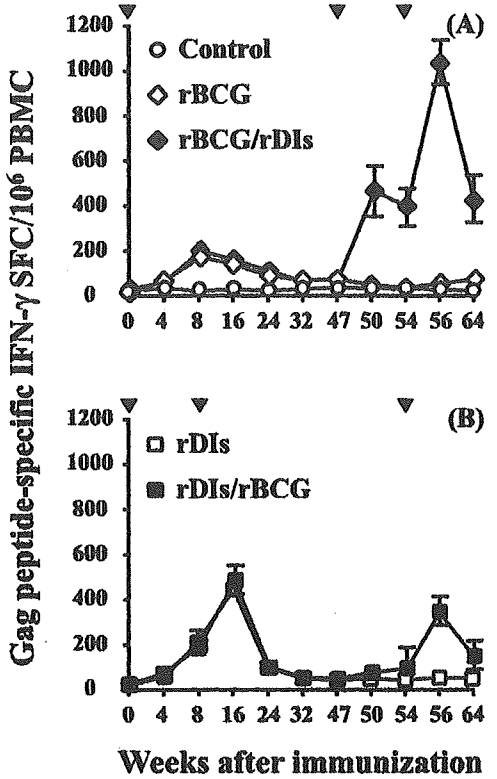

FIG. 2. Kinetics of SIV Gag peptide-specific IFN-γ spot-forming cell responses. PBMC freshly isolated from macaques immunized with either rBCG-SIVgag or rDIsSIVgag alone or with the two in combination were assessed for their ability to produce IFN-γ in response to stimulation by overlapping peptides that span the SIV Gag protein. Arrows indicate inoculation dates of the rBCG/rDIs, rBCG,and control groups, and error bars represent mean ± standard deviation.

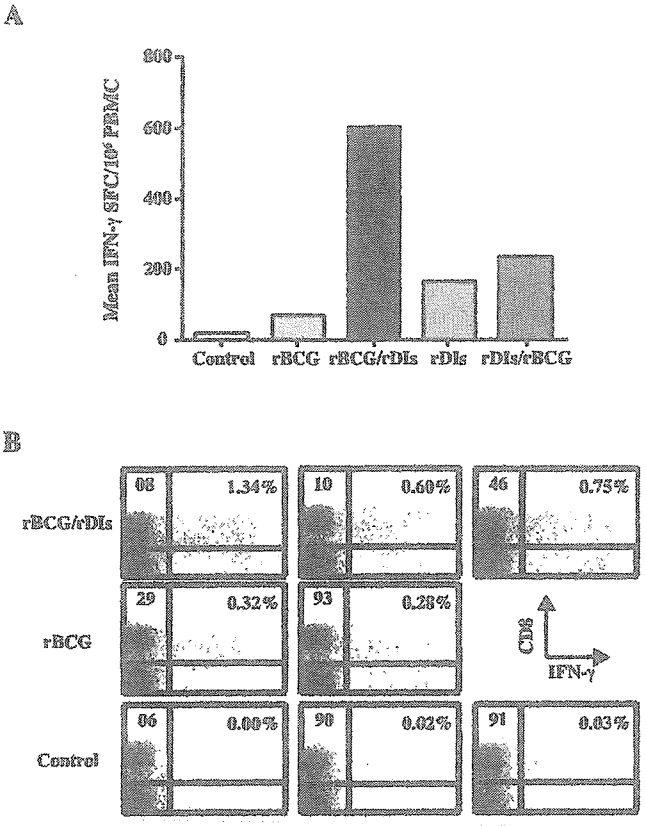

FIG. 3. SIV Gag-specific IFN-γ production in both $CD8^+$ and non-$CD8^-$ T cells in animals immunized with the rBCG/rDIs priming-boosting regimen. (A) SIV Gag protein-specific IFN-γ Elispot responses in immunized monkeys. Monkey PBMC were prepared 2 weeks after final boosting, and 2×10^5 cells were stimulated with 2 μg of recombinant SIV Gag p27 antigen protein. The bars indicate mean values of antigen-specific IFN-γ spot-forming cells per 10^6 PBMC. (B) Flow cytometric analysis of IFN-γ-producing T cells specific for SIV Gag. PBMC from macaques were cultured in vitro with overlapping peptides and stained for intracellular IFN-γ. The percentage of IFN-γ-producing $CD8^+$ T cells in each macaque's PBMC was determined by flow cytometry 2 weeks after final boosting.

responses in other rBCG and rDIs groups, both at 56 weeks postinfection and before mucosal challenge with pathogenic SHIV ($P < 0.05$). Furthermore, the number of SFC in the control and in the two naïve macaque groups did not exceed twenty during the 64-wk immunization period. Thus, the two booster inoculations of rDIs in rBCG-immunized animals effectively induced Gag peptide-specific IFN-γ-ELISPOT responses in peripheral blood, with the booster effect of DIs somewhat resembling that observed in our previous report on DNA/DIs prime-boost immunization in mice (47).

We further studied the induction of SIV Gag-specific IFN-γ ELISPOT by stimulating PBMC with SIV Gag^{p27} protein 56 weeks postinfection (Fig. 3A). The rBCG/rDIs group expressed whole-protein-specific IFN-γ responses of 615 ± 49 cells per million PBMC and the highest peptide-specific ELISPOT responses at 56 weeks postinfection (Fig. 2) of all five groups, with the peptide-specific responses being higher than the protein-specific responses (Fig. 2 and 3A). Other groups exhibited fewer than 200 cells per million PBMC.

To characterize the cellular immune responses in the rBCG/rDIs group, PBMC from the rBCG/rDIs-immunized macaques were compared with those of the rBCG and control groups by staining the surface for CD8 and intracellular SIV Gag-specific IFN-γ expression ($CD8^+IFN\text{-}\gamma^+$ cells) and then performing flow cytometric analysis (Fig. 3B). In vitro stimulation of PBMC with SIV Gag peptides in macaques 08, 10, and 46 of the rBCG/rDIs group generated a higher percentage of $CD8^+$ $IFN\text{-}\gamma^+$ T cells (1.34, 0.60, and 0.75%, respectively) than it did in animals of the rBCG group. Furthermore, non-$CD8^+$ T cells in PBMC from each animal of the rBCG/rDIs group expressed higher levels of SIV Gag-specific IFN-γ activities (macaque 8: 0.42%; macaque 10: 0.29%; macaque 46: 0.55%) than did those of the other two animal groups. The vector control animals had fewer than 0.03% of both $CD8^+$ $IFN\text{-}\gamma^+$ and non-$CD8^+$ $IFN\text{-}\gamma^+$ double-positive cells in PBMC. These findings show that the rBCG/rDIs prime-boost immunization augmented numbers of both IFN-γ-specific intracellular staining-positive cells and ELISPOT in the immunized animals, and that antigen-specific IFN-γ activities were highly induced in $CD8^+$ as well as in non-$CD8^+$ T cells, the latter most likely being $CD4^+$ T cells.

Mucosal challenge study with virulent SHIV KS661c for vaccine efficacy. Ten weeks after the second booster immunization or 64 weeks postinfection, the macaques were chal-

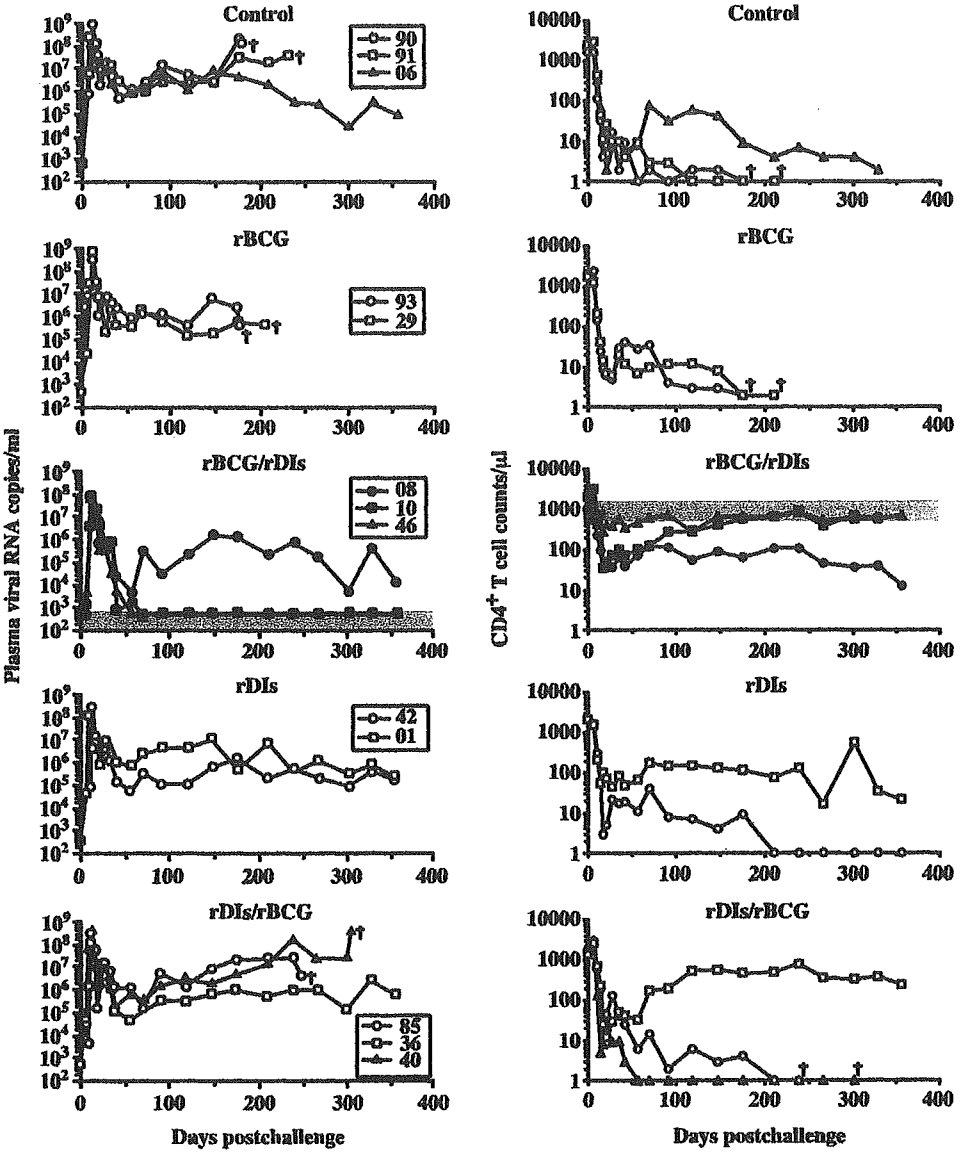

FIG. 4. Plasma viral loads and CD4 T-cell counts after viral challenge. Postchallenge plasma viral RNA copies and absolute CD4 T-cell counts in peripheral blood were detected in macaques in each of five groups immunized with a consecutive prime-boost regimen consisting of rBCG-SIVgag and rDIsSIVgag. In the study, 13 macaques were divided into five groups following the experimental designs described in Table 1.

lenged by intrarectal inoculation with 2×10^3 $TCID_{50}$ or 50 50% monkey infectious doses (MID_{50}) of SHIV KS661c, a molecular clone derived from an SHIV-89.6 variant. As shown in Fig. 4, only those macaques in the rBCG/rDIs group first primed with rBCG-SIVgag and then boosted with two inoculations of rDIsSIVgag showed evidence of protective immune responses (rBCG/rDIs). For two animals in this group (macaques 10 and 46), plasma viremia levels remained undetectable (<500 RNA copies/ml, shadow in left panel of rBCG/rDIs in Fig. 4) and $CD4^+$ T-cell counts stayed above 500 cells/μl (shadow in right panel of rBCG/rDIs in Fig. 4) for the entire year of testing. The third animal in this group (macaque 08) had fluctuating levels of viremia that were still significantly lower than those of animals in the other immunization groups. Coincidentally, this animal also had significantly decreased $CD4^+$ T-cell counts.

All macaques in the rBCG/rDIs group remained clinically healthy during the one-year observation period. Those in the rDIs/rBCG group maintained antigen-specific immune responses (Fig. 6), but showed no protective immunity against viral challenge, except for macaque 36 who showed fluctuation in the number of $CD4^+$ T cells, with numbers dipping at times below 500 cells/μl (rDIs/rBCG in Fig. 4). macaques in the other three groups all showed high levels of plasma viremia and a loss of $CD4^+$ T cells, suggesting that vaccination with rBCG and rDIs, either alone or as a priming agent, may not be suitable to induce effective, long-term positive immunity against mucosal challenge by virulent virus. By day 170 after

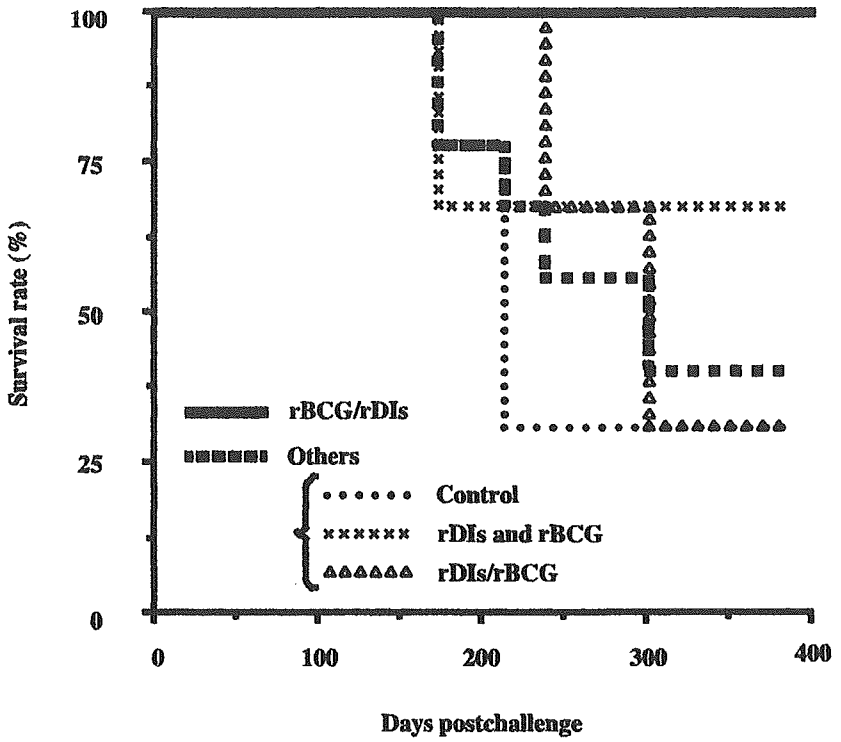

FIG. 5. Survival rates of immunized and control macaques in each of the five immunization groups. A Kaplan-Meier plot of cumulative survival rates at 1 year postchallenge with pathogenic SHIV is shown. The bold line represents group 3 immunized with the rBCG/rDIs priming-boosting regimen; the rectangular broken line represents the mean value for the total number of animals in groups 1, 2, 4, and 5.

challenge, six of the 13 macaques had died with symptoms consistent with simian AIDS: four with interstitial pneumonia, one with neurological disturbances, and one with acquired hemorrhagic diathesis. Analysis of the cumulative survival rate using the Kaplan-Meier plot showed that the rBCG/rDIs group vaccinated with the priming-boosting regimen had a superior survival rate ($P = 0.012$) to the other groups receiving vaccine protocols ($P = 0.548$) and to the control group (Fig. 5). These findings demonstrate that a prime-boost immunization with rBCG-SIVgag/rDIsSIVgag controlled virulent immunodeficiency virus infection in macaques for at least 1 year and more significantly improved survival rates than did other vaccine protocols.

Immune correlates of protection after viral challenge. In order to study virus-specific immune enhancement by SHIV challenge, we followed the postchallenge expansion of the virus-specific IFN-γ-positive cells in each animal by comparing the virus-specific IFN-γ-positive cell numbers pre- and postchallenge (Fig. 6). In all of the challenged animals of the rBCG/rDIs group, the mean number of IFN-γ-positive cells expanded from 369 ± 73 at the time of viral challenge to 629 ± 41 cells per 10^6 PBMC at 7 days after viral challenge, the sharpest increase noted with any of the animal groups. The animals of the rDIs/rBCG group showed much less enhancement, from a mean of 108 ± 46 cells per 10^6 PBMC before challenge to 224 ± 64 postchallenge, demonstrating that cellular immune responses are enhanced by viral challenge in the initial viral infection period in animals. Although in the rBCG/rDIs group high levels of IFN-γ production were observed in both $CD8^+$ and non-$CD8^+$ T cells in all three monkeys, macaque 10 and macaque 46 maintained undetectable setpoint levels of plasma viral load and normal numbers of $CD4^+$ lymphocytes, while macaque 08 did not. The macaques showed no clinical sign of weight loss, lymphoadenopathy, splenomegaly, anemia, or thrombocytopenia in the 1-year observation period. Furthermore, macaques in the rDIs group survived under low

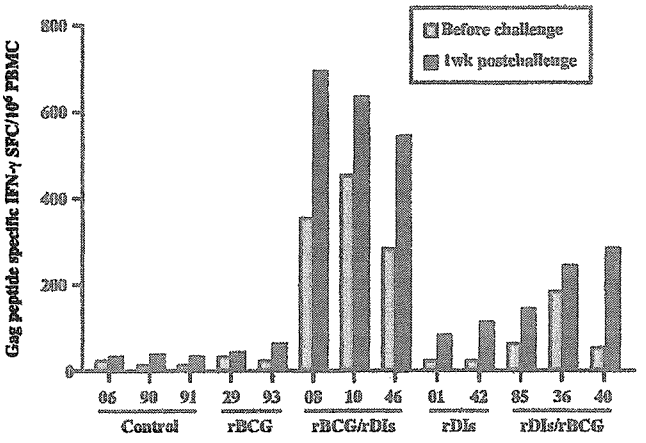

FIG. 6. Virus challenge enhances the SIV Gag peptide-specific IFN-γ ELISPOTs in PBMC from immunized macaques. PBMC from immunized animals were tested with pools of peptides spanning all the proteins from SIVmac239. Results show the production of IFN-γ to pooled peptides in $CD8^+$ and non-$CD8^-$ T lymphocytes.

immune induction but exhibited $CD4^+$ T-cell loss and plasma viremia. Notably, all macaques in the control group exhibited very low levels of immune induction by viral challenge and showed no viral control.

DISCUSSION

In the current study, we initially produced rBCG expressing SIV whole Gag. Second, by introducing a priming-boosting regimen combining rBCG-SIVgag with a nonreplicating rDIsSIVgag, we found that the rBCG/rDIs vaccination induced a long-lasting and effective immunity that was able to control a highly pathogenic virus after mucosal challenge in macaques. Third, elicitation of virus-specific immunity was observed to be important in exerting viral control in the animals immunized with the prime-boost vaccine regimen. Further investigation using larger groups of animals will be needed to determine whether high levels of immune induction correlate with increased efficacy. In this study, the macaques in the rBCG/rDIs group developed high levels of cellular immunity and were protected against the loss of $CD4^+$ T lymphocytes and the increase of viral RNA levels induced by viral challenge. Furthermore, the rBCG/rDIs group showed no evidence of clinical diseases or mortality after viral challenge during the 1-year period of observation.

The rBCG/rDIs prime-boost vaccine controlled the infection efficiently for the duration of the one-year observation period, reducing viral loads to below the threshold level for RNA copies in peripheral blood and maintaining the $CD4^+$ cell numbers above 500 cells per microliter of peripheral blood in two of the three animals in group 3. The remaining animal in the group showed fluctuations in the two parameters. Viral loads and $CD4^+$ cell numbers were not significantly affected in animal groups following the other vaccine regimens. The level of vaccine efficacy for the rBCG/rDIs group seems to be comparable to that observed in previous studies with DNA/fusion protein of interleukin-2 and immunoglobulin G (5), DNA/MVA (3), DNA/recombinant adenovirus type 5 (Ad5) (45), and MVA/recombinant vesicular stomatitis virus (36); that is, effective control of pathogenic SHIV 89.6P infection was achieved in macaques for 6 to 8 months.

SHIV KS661c, which was used as a mucosal challenge virus in this study, is a highly pathogenic molecular clone of a variant of SHIV-89.6 possessing a tropism of CXCR-4. In our preliminary study, the SHIV virus infected GHOST-X4 cells in vitro and the virus challenge eliminated the naïve $CD4^+$ T-cell population in the peripheral blood in macaques, findings which confirmed those by Nishimura et al. (33, 34). In conjunction with CCR5-tropic pathogenic SIVsmE660, Ourmanov et al. obtained similar results with the partial control of homologous viremia by the recombinant MVA vaccination (35). Furthermore, the potential of the DNA vaccination to induce a broad spectrum of mucosal protection against heterologous SIV/DeltaB670 has been demonstrated (13).

Although the virus-specific immune elicitation by DNA/Ad5 vaccination was extremely high in immunized animals (45), the efficacy results for a DNA/Ad5 study with an SIVmac239 were not comparable to those for SHIV 89.6 (43). These discrepancies in vaccine efficacy by challenge viruses suggest that SIVmac239 might be a difficult virus to control by the active immunization of various vaccine candidates. Since DNA/Ad5 is expected to elicit higher levels of immunity than either MVA or DNA alone (43–45), it might be possible to obtain vaccine efficacy in conjunction with different CCR5-tropic SIV or SHIV from SIVmac239. Alternatively, a multicomponent DNA/Ad5 might elicit broad-spectrum immunity as well as protection against SIV or CCR5-SHIV. Recently, a DNA/Sendai virus vaccination (27) proved to be as effective at controlling SIVmac239 as an attenuated live SIV vaccine (10, 11), opening the possibility for studies comparing the protective immunity elicited by ordinary vaccination to that induced by attenuated live SIV vaccination.

Because the lack of an exact HIV-1 macaque model significantly limits our ability to study and calibrate vaccine efficacy, we may need to rely on parameters such as the control of viremia, the loss of $CD4^+$ cells, and the absence of mortality to establish the efficacy of a tested vaccine against an immunodeficiency virus. Certainly, such parameters would represent a more realistic goal for the development of a preventive vaccine in the macaque model. They may also play a key role in the evaluation of vaccine efficacy in human trials I/II using the vaccine modalities developed in the macaque model.

It was recently reported that the AIDS vaccine failed in rhesus macaques approximately six months post-virus challenge, with viral avoidance of cytotoxic T-lymphocyte recognition posing a major limitation to cytotoxic T-lymphocyte-based AIDS vaccines (4). In contrast, the rBCG/rDIs prime-boost vaccine was shown in this study to control viral load throughout the 1-year observation period, suggesting that it may improve the prospects for a vaccine regimen capable of providing long-term protection against HIV-1 replication and disease progression (38, 49). Work is under way to determine whether this rBCG/rDIs vaccine will fail to control the plasma viral load in the macaque model, a failure associated with the viral escape of antigen-specific cytotoxic T lymphocytes.

The route of recombinant DIs administration will be key to effectively inducing immunity in humans. In the preliminary study to determine cellular immune induction, hundred times more rDIs was needed to achieve SIV Gag antigen-specific immunity in macaques by the intradermal (10^8 PFU/ml) than by the intravenous (10^6 PFU/ml) route (K. Someya et al., unpublished data). These findings may suggest that replication-defective vaccinia virus DIs is effective at eliciting antigen-specific immunity by intravenous administration. In addition, they suggest that the intravenous inoculation of rDIs may more effectively induce specific immunity than intradermal inoculation, although intravenous inoculation is not practical for use in human.

This study did not show a clear correlation between levels of virus-specific cellular immunity induced by booster inoculations with rDIs to rBCG-primed animals and protection against a highly virulent immunodeficiency virus after mucosal challenge. The levels of both virus-specific IFN-γ ELISPOT and gamma interferon cytokine staining responses in peripheral blood from animals in the rBCG/rDIs group were the highest of the five groups studied. Why did the prime-boost vaccination of animals of the rBCG/rDIs group prove more effective than the vaccine protocols used with the other groups? We speculate that rBCG priming, which occurs at the skin region of the thigh near the inguinal and iliac lymph nodes

draining the genitorectal mucosa, may elicit mucosal immunity in the region (23). Furthermore, we showed that the two booster intravenous inoculations with rDIs help induce a level of protective immunity sufficient to control a mucosal viral challenge in the immunized animals. Although the two intravenous inoculations with rDIsSIVgag alone proved capable of inducing some virus-specific immunity in peripheral blood after the homologous booster immunization in the immunized animals (DIs group), they appeared to provide no protection against the mucosal viral challenge.

The *M. bovis* BCG/DIs prime-boost vaccination might thus provide the opportunity to study the relationship between protection against mucosal viral challenge and elicitation of systemic or mucosal immunity. Our findings regarding the efficacy of the *M. bovis* BCG/DIs prime-boost vaccine regimen confirm those by Lehner et al. (23) and they further demonstrated a significant association between protection from mucosal rectal infection with SIV and an increase in the levels of CD8 suppressor factor and beta-chemokine. Although we cannot fully explain the differences in vaccine efficacy at this moment, it is likely that the routes of immunization and of challenge, the character of the vaccine vectors and the immunization schedule all play profound roles in eliciting vaccine efficacy in macaques.

Recently, considerable progress has been made in understanding *M. bovis* BCG as a HIV vaccine vector. Our own group demonstrated that recombinant *M. bovis* BCG vectors have the potential to deliver an HIV immunogen for desirable immune elicitation in macaques (46). Furthermore, *M. bovis* BCG vaccine substrain Tokyo 172 was revealed to be avirulent in HIV-infected children (16). The insertion of a full-length SIVmac239 *gag* into the *M. bovis* BCG substrain Tokyo 172 does not affect its toxicity, stability, or efficacy against *Mycobacterium tuberculosis* (54). Furthermore, rBCG has been shown to be nonvirulent in immunodeficient mice (54). These findings highlight the utility of rBCG as a vector for HIV-1 vaccine development.

In summary, our results demonstrate that a prime-boost vaccine regimen using rBCG as the prime and vaccinia virus rDIs as the boost can induce effective immunity against a mucosal infection with a highly virulent immunodeficiency virus for at least a year. Both of the vectors are safe for humans, making them attractive candidates for use in a preventive prime-boost vaccine against HIV-1.

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REFERENCES

1. **Aldovini, A., and R. A. Young.** 1991. Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. Nature 351:479–482.
2. **Amara, R. R., and H. L. Robinson.** 2002. A new generation of HIV vaccines. Trends Mol. Med. 8:489–495.
3. **Amara, R. R., F. Villinger, J. D. Altman, S. L. Lydy, S. P. O'Neil, S. I. Staprans, D. C. Montefiori, Y. Xu, J. G. Herndon, L. S. Wyatt, M. A. Candido, N. L. Kozyr, P. L. Earl, J. M. Smith, H. L. Ma, B. D. Grimm, M. L. Hulsey, J. Miller, H. M. McClure, J. M. McNicholl, B. Moss, and H. L. Robinson.** 2001. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Science 292:69–74.
4. **Barouch, D. H., J. Kunstman, M. J. Kuroda, J. E. Schmitz, S. Santra, F. W. Peyerl, G. R. Krivulka, K. Beaudry, M. A. Lifton, D. A. Gorgone, D. C. Montefiori, M. G. Lewis, S. M. Wolinsky, and N. L. Letvin.** 2002. Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. Nature 415:335–339.
5. **Barouch, D. H., S. Santra, J. E. Schmitz, M. J. Kuroda, T. M. Fu, W. Wagner, M. Bilska, A. Craiu, X. X. Zheng, G. R. Krivulka, K. Beaudry, M. A. Lifton, C. E. Nickerson, W. L. Trigona, K. Punt, D. C. Freed, L. Guan, S. Dubey, D. Casimiro, A. Simon, M. E. Davies, M. Chastain, T. B. Strom, R. S. Gelman, D. C. Montefiori, M. G. Lewis, E. A. Emini, J. W. Shiver, and N. L. Letvin.** 2000. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. Science 290:486–492.
6. **Chaparas, S. D., and S. R. Hedrick.** 1973. Comparison of strains of BCG. I. Antigenic analysis and tuberculin reactivity. Infect. Immun. 7:777–780.
7. **Chujoh, Y., K. Matsuo, H. Yoshizaki, T. Nakasatomi, K. Someya, Y. Okamoto, S. Naganawa, S. Haga, H. Yoshikura, A. Yamazaki, S. Yamazaki, and M. Honda.** 2001. Cross-clade neutralizing antibody production against human immunodeficiency virus type 1 clade E and B' strains by recombinant *Mycobacterium bovis* BCG-based candidate vaccine. Vaccine 20:797–804.
8. **Daniel, M. D., F. Kirchhoff, S. C. Czajak, P. K. Sehgal, and R. C. Desrosiers.** 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. Science 258:1938–1941.
9. **Davis, N. L., A. West, E. Reap, G. MacDonald, M. Collier, S. Dryga, M. Maughan, M. Connell, C. Walker, K. McGrath, C. Cecil, L. H. Ping, J. Frelinger, R. Olmsted, P. Keith, R. Swanstrom, C. Williamson, P. Johnson, D. Montefiori, and R. E. Johnston.** 2002. Alphavirus replicon particles as candidate HIV vaccines. IUBMB Life 53:209–211.
10. **Desrosiers, R. C.** 1992. HIV with multiple gene deletions as a live attenuated vaccine for AIDS. AIDS Res. Hum. Retroviruses 8:1457.
11. **Desrosiers, R. C., M. S. Wyand, T. Kodama, D. J. Ringler, L. O. Arthur, P. K. Sehgal, N. L. Letvin, N. W. King, and M. D. Daniel.** 1989. Vaccine protection against simian immunodeficiency virus infection. Proc. Natl. Acad. Sci. USA 86:6353–6357.
12. **Fauci, A. S., S. M. Schnittman, G. Poli, S. Koenig, and G. Pantaleo.** 1991. NIH conference on immunopathogenic mechanisms in human immunodeficiency virus infection. Ann. Intern. Med. 114:678–693.
13. **Fuller, D. H., P. A. Rajakumar, L. A. Wilson, A. M. Trichel, J. T. Fuller, T. Shipley, M. S. Wu, K. Weis, C. R. Rinaldo, J. R. Haynes, and M. Murphey-Corb.** 2002. Induction of mucosal protection against primary, heterologous simian immunodeficiency virus by a DNA vaccine. J. Virol. 76:3309–3317.
14. **Gauduin, M. C., R. L. Glickman, S. Ahmad, T. Yilma, and R. P. Johnson.** 1999. Characterization of SIV-specific $CD4^+$ T-helper proliferative responses in macaques immunized with live-attenuated SIV. J. Med. Primatol. 28:233–241.
15. **Hel, Z., D. Venzon, M. Poudyal, W. P. Tsai, L. Giuliani, R. Woodward, C. Chougnet, G. Shearer, J. D. Altman, D. Watkins, N. Bischofberger, A. Abimiku, P. Markham, J. Tartaglia, and G. Franchini.** 2000. Viremia control following antiretroviral treatment and therapeutic immunization during primary SIV251 infection of macaques. Nat. Med. 6:1140–1146.
16. **Hesseling, A. C., H. S. Schaaf, W. A. Hanekom, N. Beyers, M. F. Cotton, R. P. Gie, B. J. Marais, P. van Helden, and R. M. Warren.** 2003. Danish bacille Calmette-Guerin vaccine-induced disease in human immunodeficiency virus-infected children. Clin. Infect. Dis. 37:1226–1233.
17. **Honda, M., K. Matsuo, T. Nakasone, Y. Okamoto, H. Yoshizaki, K. Kitamura, W. Sugiura, K. Watanabe, Y. Fukushima, S. Haga, Y. Katsura, K. Kitamura, H. Tasaka, K. Komuro, T. Yamada, T. Asano, A. Yamazaki, and S. Yamazaki.** 1995. Protective immune responses induced by secretion of a chimeric soluble protein from a recombinant *Mycobacterium bovis* bacillus Calmette-Guérin vector candidate vaccine for human immunodeficiency virus type 1 in small animals. Proc. Natl. Acad. Sci. USA 92:10693–10697.
18. **Ishii, K., Y. Ueda, K. Matsuo, Y. Matsuura, T. Kitamura, K. Kato, Y. Izumi, K. Someya, T. Ohsu, M. Honda, and T. Miyamura.** 2002. Structural analysis of vaccinia virus DIs strain: application as a new replication-deficient viral vector. Virology 302:433–444.
19. **Jacobs, W. R., Jr., M. Tuckman, and B. R. Bloom.** 1987. Introduction of foreign DNA into mycobacteria using a shuttle phasmid. Nature 327:532–535.
20. **Karacostas, V., E. J. Wolffe, K. Nagashima, M. A. Gonda, and B. Moss.** 1993. Over expression of the HIV-1 gag-pol polyprotein results in intracellular

activation of HIV-1 protease and inhibition of assembly and budding of virus-like particles. Virology 193:661–671.
21. Kawahara, M., K. Matsuo, T. Nakasone, T. Hiroi, H. Kiyono, S. Matsumoto, T. Yamada, N. Yamamoto, and M. Honda. 2002. Combined intrarectal/intradermal inoculation of recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) induces enhanced immune responses against the inserted HIV-1 V3 antigen. Vaccine 21:158–166.
22. Kitamura, T., Y. Kitamura, and I. Tagaya. 1967. Immunogenicity of an attenuated strain of vaccinia virus on rabbits and monkeys. Nature 215:1187–1188.
23. Lehner, T., Y. Wang, M. Cranage, L. A. Bergmeier, E. Mitchell, L. Tao, G. Hall, M. Dennis, N. Cook, R. Brookes, L. Klavinskis, I. Jones, C. Doyle, and R. Ward. 1996. Protective mucosal immunity elicited by targeted iliac lymph node immunization with a subunit SIV envelope and core vaccine in macaques. Nat. Med. 2:767–775.
24. Letvin, N. L., D. H. Barouch, and D. C. Montefiori. 2002. Prospects for vaccine protection against HIV-1 infection and AIDS. Annu. Rev. Immunol. 20:73–99.
25. Lifson, J. D., M. Piatak, Jr., J. L. Rossio, J. Bess, Jr., E. Chertova, D. Schneider, R. Kiser, V. Coalter, B. Poore, R. Imming, R. C. Desrosiers, L. E. Henderson, and L. O. Arthur. 2002. Whole inactivated SIV virion vaccines with functional envelope glycoproteins: safety, immunogenicity, and activity against intrarectal challenge. J. Med. Primatol. 31:205–216.
26. Lu, Y., M. S. Salvato, C. D. Pauza, J. Li, J. Sodroski, K. Manson, M. Wyand, N. Letvin, S. Jenkins, N. Touzjian, C. Chutkowski, N. Kushner, M. LeFaile, L. G. Payne, and B. Roberts. 1996. Utility of SHIV for testing HIV-1 vaccine candidates in macaques. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 12:99–106.
27. Matano, T., M. Kobayashi, H. Igarashi, A. Takeda, H. Nakamura, M. Kano, C. Sugimoto, K. Mori, A. Iida, T. Hirata, M. Hasegawa, T. Yuasa, M. Miyazawa, Y. Takahashi, M. Yasunami, A. Kimura, D. H. O'Connor, D. I. Watkins, and Y. Nagai. 2004. Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. J. Exp. Med. 199:1709–1718.
28. Matsumoto, S., M. Tamaki, H. Yukitake, T. Matsuo, N. Mariko, H. Teraoka, and T. Yamada. 1996. A stable *Escherichia coli*-mycobacteria shuttle vector pSO246 in *Mycobacterium bovis* BCG. FEMS Microbiol. Lett. 135:237–243.
29. McMichael, A. J., and T. Hanke. 2003. HIV vaccines 1983–2003. Nat. Med. 9:874–880.
30. Mori, K., Y. Yasutomi, S. Ohgimoto, T. Nakasone, S. Takamura, T. Shioda, and Y. Nagai. 2001. Quintuple deglycosylation mutant of simian immunodeficiency virus SIVmac239 in rhesus macaques: robust primary replication, tightly contained chronic infection, and elicitation of potent immunity against the parental wild-type strain. J. Virol. 75:4023–4028.
31. Mothe, B. R., H. Horton, D. K. Carter, T. M. Allen, M. E. Liebl, P. Skinner, T. U. Vogel, S. Fuenger, K. Vielhuber, W. Rehrauer, N. Wilson, G. Franchini, J. D. Altman, A. Haase, L. J. Picker, D. B. Allison, and D. I. Watkins. 2002. Dominance of CD8 responses specific for epitopes bound by a single major histocompatibility complex class I molecule during the acute phase of viral infection. J. Virol. 76:875–884.
32. Nabel, G., W. Makgoba, and J. Esparza. 2002. HIV-1 diversity and vaccine development. Science 296:2335.
33. Nishimura, Y., C. R. Brown, J. J. Mattapallil, T. Igarashi, A. Buckler-White, B. A. Lafont, V. M. Hirsch, M. Roederer, and M. A. Martin. 2005. Resting naive CD4+ T cells are massively infected and eliminated by X4-tropic simian-human immunodeficiency viruses in macaques. Proc. Natl. Acad. Sci. USA 102:8000–8005.
34. Nishimura, Y., T. Igarashi, O. K. Donau, A. Buckler-White, C. Buckler, B. A. Lafont, R. M. Goeken, S. Goldstein, V. M. Hirsch, and M. A. Martin. 2004. Highly pathogenic SHIVs and SIVs target different CD4+ T-cell subsets in rhesus monkeys, explaining their divergent clinical courses. Proc. Natl. Acad. Sci. USA 101:12324–12329.
35. Ourmanov, I., C. R. Brown, B. Moss, M. Carroll, L. Wyatt, L. Pletneva, S. Goldstein, D. Venzon, and V. M. Hirsch. 2000. Comparative efficacy of recombinant modified vaccinia virus Ankara expressing simian immunodeficiency virus (SIV) Gag-Pol and/or Env in macaques challenged with pathogenic SIV. J. Virol. 74:2740–2751.
36. Ramsburg, E., N. F. Rose, P. A. Marx, M. Mefford, D. F. Nixon, W. J. Moretto, D. Montefiori, P. Earl, B. Moss, and J. K. Rose. 2004. Highly effective control of an AIDS virus challenge in macaques by using vesicular stomatitis virus and modified vaccinia virus Ankara vaccine vectors in a single-boost protocol. J. Virol. 78:3930–3940.
37. Reimann, K. A., J. T. Li, G. Voss, C. Lekutis, K. Tenner-Racz, P. Racz, W. Lin, D. C. Montefiori, D. E. Lee-Parritz, Y. Lu, R. G. Collman, J. Sodroski, and N. L. Letvin. 1996. An env gene derived from a primary human immunodeficiency virus type 1 isolate confers high in vivo replicative capacity to a chimeric simian/human immunodeficiency virus in rhesus monkeys. J. Virol. 70:3198–3206.
38. Ruprecht, R. M., T. W. Baba, R. Rasmussen, Y. Hu, and P. L. Sharma. 1996. Murine and simian retrovirus models: the threshold hypothesis. AIDS 10(Suppl. A):S33–40.
39. Sakai, K., K. Shinohara, E. Takahashi, Y. Izumi, Y. Ami, Y. Sasaki, T. Nakasone, and M. Honda. 2001. Molecular cloning of a pathogenic simian-human immunodeficiency virus for HIV/AIDS monkey model. Sixth International Congress on AIDS in Asia and the Pacific, 5–10 October 2001, Melbourne, Australia, abstract p. 84.
40. Sasaki, Y., Y. Ami, T. Nakasone, K. Shinohara, E. Takahashi, S. Ando, K. Someya, Y. Suzaki, and M. Honda. 2000. Induction of CD95 ligand expression on T lymphocytes and B lymphocytes and its contribution to apoptosis of CD95-up-regulated $CD4^-$ T lymphocytes in macaques by infection with a pathogenic simian/human immunodeficiency virus. Clin. Exp. Immunol. 122:381–389.
41. Sher, N. A., S. D. Chaparas, L. E. Greenberg, and S. Bernard. 1975. Effects of BCG, *Corynebacterium parvum*, and methanol extraction residue in the reduction of mortality from *Staphylococcus aureus* and *Candida albicans* infections in immunosuppressed mice. Infect. Immun. 12:1325–1330.
42. Shinohara, K., K. Sakai, S. Ando, Y. Ami, N. Yoshino, E. Takahashi, K. Someya, Y. Suzaki, T. Nakasone, Y. Sasaki, M. Kaizu, Y. Lu, and M. Honda. 1999. A highly pathogenic simian/human immunodeficiency virus with genetic changes in cynomolgus monkey. J. Gen. Virol. 80:1231–1240.
43. Shiver, J. W. 2004. Development of an HIV-1 vaccine based on replication-defective adenovirus. Proc. Keystone Symp. 2004:12.
44. Shiver, J. W., and E. A. Emini. 2004. Recent advances in the development of HIV-1 vaccines using replication-incompetent adenovirus vectors. Annu. Rev. Med. 55:355–372.
45. Shiver, J. W., T. M. Fu, L. Chen, D. R. Casimiro, M. E. Davies, R. K. Evans, Z. Q. Zhang, A. J. Simon, W. L. Trigona, S. A. Dubey, L. Huang, V. A. Harris, R. S. Long, X. Liang, L. Handt, W. A. Schleif, L. Zhu, D. C. Freed, N. V. Persaud, L. Guan, K. S. Punt, A. Tang, M. Chen, K. A. Wilson, K. B. Collins, G. J. Heidecker, V. R. Fernandez, H. C. Perry, J. G. Joyce, K. M. Grimm, J. C. Cook, P. M. Keller, D. S. Kresock, H. Mach, R. D. Troutman, L. A. Isopi, D. M. Williams, Z. Xu, K. E. Bohannon, D. B. Volkin, D. C. Montefiori, A. Miura, G. R. Krivulka, M. A. Lifton, M. J. Kuroda, J. E. Schmitz, N. L. Letvin, M. J. Caulfield, A. J. Bett, R. Youil, D. C. Kaslow, and E. A. Emini. 2002. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature 415:331–335.
46. Someya, K., D. Cecilia, Y. Ami, T. Nakasone, K. Matsuo, S. Burda, H. Yamamoto, N. Yoshino, M. Kaizu, S. Ando, K. Okuda, S. Zolla-Pazner, S. Yamazaki, N. Yamamoto, and M. Honda. 2005. Vaccination of rhesus macaques with recombinant *Mycobacterium bovis* bacillus Calmette-Guérin Env V3 elicits neutralizing antibody-mediated protection against simian-human immunodeficiency virus with a homologous but not a heterologous V3 motif. J. Virol. 79:1452–1462.
47. Someya, K., K. Q. Xin, K. Matsuo, K. Okuda, N. Yamamoto, and M. Honda. 2004. A consecutive priming-boosting vaccination of mice with simian immunodeficiency virus (SIV) *gag/pol* DNA and recombinant vaccinia virus strain DIs elicits effective anti-SIV immunity. J. Virol. 78:9842–9853.
48. Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, W. R. Jacobs, and B. R. Bloom. 1991. New use of BCG for recombinant vaccines. Nature 351:456–460.
49. Strathdee, S. A., K. J. Craib, R. S. Hogg, M. V. O'Shaughnessy, J. S. Montaner, and M. T. Schechter. 1995. Long-term non-progression in HIV infection. Lancet 346:1372.
50. Tagaya, I., T. Kitamura, and Y. Sano. 1961. A new mutant of dermovaccinia virus. Nature 192:381–382.
51. Takeya, K., K. Nomoto, S. Muraoka, S. Shimotori, T. Taniguchi, and T. Miyake. 1977. Growth of two strains of *Mycobacterium bovis* (BCG) in a thymic mice. J. Gen. Microbiol. 100:403–405.
52. Thole, J. E., W. J. Keulen, J. De Bruyn, A. H. Kolk, D. G. Groothuis, L. G. Berwald, R.H. Tiesjema, and J. D. van Embden. 1987. Characterization, sequence determination, and immunogenicity of a 64-kilodalton protein of *Mycobacterium bovis* BCG expressed in *Escherichia coli* K-12. Infect. Immun. 55:1466–1475.
53. Ueda, K., S. Yamazaki, J. Saegusa, and S. Someya. 1978. Progressive and fetal infection with attenuated *Mycobacterium bovis* (BCG) in nude mice. Jpn. J. Exp. Med. 48:533–543.
54. Yamamoto, S., T. Yamamoto, S. Haga, and M. Honda. 2003. Presented at the Tuberculosis Leprosy Research Conference U.S.-Japan Co. Med. Sci.
55. Yoshino, N., Y. Ami, K. Terao, F. Tashiro, and M. Honda. 2000. Upgrading of flow cytometric analysis for absolute counts, cytokines and other antigenic molecules of cynomolgus monkeys (*Macaca fascicularis*) by using anti-human cross-reactive antibodies. Exp. Anim. 49:97–110.

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Enterotoxin-Based Mucosal Adjuvants Alter Antigen Trafficking and Induce Inflammatory Responses in the Nasal Tract

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The safety of nasal vaccines containing enterotoxin-based mucosal adjuvants has not been studied in detail. Previous studies have indicated that native cholera toxin (nCT) can alter antigen trafficking when applied nasally. In this study, we determined the enterotoxin-based variables that alter antigen trafficking. To measure the influence of enterotoxin-based mucosal adjuvants on antigen trafficking in the nasal tract, native and mutant enterotoxins were coadministered with radiolabeled tetanus toxoid (TT). The nCT and heat-labile enterotoxin type 1 (LTh-1) redirected TT into the olfactory neuroepithelium (ON/E). Antigen redirection occurred mainly across the nasal epithelium without subsequent transport along olfactory neurons into the olfactory bulbs (OB). Thus, no significant accumulation of the vaccine antigen TT was observed in the OB when coadministered with nCT. In contrast, neither mutant CT nor mutant LTh-1, which lack ADP-ribosyltransferase activity, redirected TT antigen into the ON/E. Thus, ADP-ribosyltransferase activity was essential for antigen trafficking across the olfactory epithelium. Accumulation of TT in the ON/E was also due to B-subunit binding to GM1 gangliosides, as was demonstrated (i) by redirection of TT by LTh-1 in a dose-dependent manner, (ii) by ganglioside inhibition of the antigen redirection by LTh-1 and nCT, and (iii) by the use of LT-IIb, a toxin that binds to gangliosides other than GM1. Redirection of TT into the ON/E coincided with elevated production of interleukin 6 (IL-6) but not IL-1β or tumor necrosis factor alpha in the nasal mucosa. Thus, redirection of TT is dependent on ADP-ribosyltransferase activity and GM1 binding and is associated with production of the inflammatory cytokine IL-6.

Enterotoxins are powerful mucosal adjuvants; however, the mechanisms for their adjuvanticity are still being defined. Native cholera toxin (nCT) and the *Escherichia coli*-derived heat-labile toxin (human type 1) (nLTh-1) are both potent mucosal adjuvants for coadministered protein antigens when given by the oral, nasal, or parenteral route (4, 9–11, 33, 42, 50). Despite extensive research on these enterotoxins, mucosal adjuvants for human use remain in experimental phases, and recent studies have focused on generating nontoxic mutants of CT (mCT) and LTh-1 (mLTh-1). Detoxification of these enterotoxins was accomplished by site-directed mutagenesis of the ADP-ribosylation site located in the A subunit of these AB_5 enterotoxins (3, 8, 51, 52, 53, 54). These mutants are effective mucosal adjuvants in mice and induce long-term memory for coadministered proteins given either by the nasal or parenteral route (3, 51, 52). In this regard, the nasal route is perhaps superior to oral delivery, since it requires much lower doses of both adjuvant and coadministered proteins/vaccines.

Both nCT and nLTh-1 are part of serogroup I of the heat-labile enterotoxins (38) and display somewhat different ganglioside binding specificities (12). For example, nCT binds predominantly to GM1, while nLTh-1 preferentially binds to GM1 and to a lesser extent to GD1b and binds weakly to GM2 and asialo-GM1 (12). Native LTh-I not only targets gangliosides, but also binds to other glycoproteins in the intestinal tract and is associated with a much larger repertoire of target molecules than has been reported for CT (18, 25). The heat-labile enterotoxins from serogroup II, such as LT-IIb, display different ganglioside binding specificities. LT-IIb binds to GD1a and to a lesser extent to GT1b and showed no affinity for GM1 (12). LT-IIb functioned as a mucosal adjuvant when given nasally and induced a mucosal immune response consistent with a mixed $CD4^+$ Th1/Th2 cell response (34), as was previously reported for nLTh-1 (42). A lack of ganglioside binding, which was accomplished by site-directed mutagenesis of amino acid 33, the G33D mutation, rendered both nCT and nLTh-1 deficient in GM1 binding and in the ability to function as mucosal adjuvants following oral (21) or nasal (7) application. Enterotoxin binding to gangliosides is functionally important for both mucosal adjuvanticity and enterotoxicity. Both nCT and nLTh-1 bind to GM1 on epithelial cells and are endocytosed and transported. Blocking GM1 sites is not sufficient to ameliorate the enterotoxicity of nLTh-1, since the molecule also binds to other intestinal epithelial glycoproteins (26, 55).

ADP-ribosyltransferase activity in nCT may potentially cause damage due to toxicity and inflammation of the nasal epithelium, and in so doing may allow passive entry of codelivered vaccine proteins into the olfactory nerve/epithelium (ON/E) (14). Increased permeability of the gut epithelium for

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low-molecular-weight dextran was seen when nCT was orally administered. This study suggested that increased permeability may be an intricate part of the ability of nCT to function as a mucosal adjuvant (32). This conclusion was supported by the fact that CT-B, which is a poor oral adjuvant, did not cause permeability changes in the gut epithelium (32).

The hypothesis proposed in this study was that part of the adjuvanticity of enterotoxin-based mucosal adjuvants may reflect their ability to alter antigen trafficking in the nasal tract and that this antigen redirection could contribute to enhanced inflammatory reactions, which may differentially boost mucosal immune responses.

In this study, we show that enterotoxin-based mucosal adjuvants, i.e., nCT and nLTh-1, alter codelivered protein vaccine trafficking into the ON/E when given nasally. This process of antigen redirection requires ADP-ribosyltransferase activity of the enterotoxin-based adjuvant, as well as binding to GM1 gangliosides, and coincided with the production of the inflammatory cytokine interleukin 6 (IL-6). On the other hand, mutants of CT and LTh-1 lacking ADP-ribosyltransferase activity did not redirect antigen into the ON/E, nor did the native toxin LT-IIb, which is not able to bind GM1. In conclusion, both ADP-ribosyltransferase activity and GM1 binding are required in order for enterotoxin to redirect antigen into the ON/E.

MATERIALS AND METHODS

Mice. Mice of the C57BL/6 strain 6 to 7 weeks of age were obtained from Charles River Laboratories (Wilmington, MA). The mice were maintained in horizontal laminar flow cabinets and were pathogen free as determined by plasma antibody screening and tissue histopathology performed on sentinel mice. All mice received sterile food and water ad libitum and were between 8 and 12 weeks of age when used for these experiments. All mouse studies were done in accordance with guidelines of both the NIH and the Animal Institutional Care and Use Committee of the University of Alabama at Birmingham to avoid pain and distress.

Enterotoxin production and purification. The enterotoxins were produced in our laboratory, with the exception of nCT, which was purchased (List Biological Laboratories, Inc., Campbell, CA). The mCT (E112K) was generated by site-directed mutagenesis of single-stranded DNA of *Escherichia coli* CJ236 transfected with M13 mp19, which included the CT gene, using the Mutant K system (Takara Biomedicals, Kyoto, Japan) as described previously (29, 53). The glutamate-to-lysine mutation of amino acid 112 was generated using the 5′-GATG AACAAAAAGTTTCTGCT-3′ oligonucleotide (53). The pUC119 plasmid carrying the mutated CT gene was transformed into *E. coli* DH5α. The *E. coli* strains containing the mCT gene were grown in LB broth (10 g NaCl, 10 g tryptone, and 5 g yeast extract/liter) with 100 μg/ml of ampicillin. The resulting mCT, derived from a sonicated cell suspension, was purified by binding to and elution from a D-galactose-immobilized column (Pierce Chemical Co., Rockford, IL).

The enterotoxin gene containing plasmid pMY1900 from *E. coli* strain 1032 was subcloned by PCR into the expression vector pTrc 99A (Amersham Pharmacia Biotech, Piscataway, NJ). The LTh-1 mutant E112K was constructed by site-directed mutagenesis with specific primers as described previously (43, 44). The mLT (E112K) and LTh-1 were purified from sonicated cell suspensions and resuspended in 0.9% NaCl in 10 mM Tris-HCl buffer, pH 8.6. After centrifugation, the supernatant was subjected to a 65% ammonium sulfate precipitation, resuspended in 0.2 M Tris (pH 8.0)–1 M sucrose–10 mM EDTA (TEAN) buffer, and purified on an immobilized D-galactose column (Pierce Chemical Co.) as reported previously (45).

Heat-labile enterotoxin IIb (LT-IIb) was produced with plasmid pTDC101-transformed *E. coli* XL-1 Blue (Stratagene, La Jolla, CA) (5). The recombinant *E. coli* was grown at 37°C with vigorous shaking (225 rpm) in Luria broth (Difco Laboratories, Detroit, MI) supplemented with ampicillin (150 μg/ml; Sigma-Aldrich, St. Louis, MO) in the presence of kanamycin (50 μg/ml; Sigma-Aldrich). The expression of LT-IIb was induced during mid-log phase by the addition of 1 mM isopropyl-β-D-thiogalactoside (Sigma-Aldrich). After 4 h of growth, the bacteria were harvested by centrifugation at 8,000 × *g* for 15 min and resuspended in ice-cold 100 mM Tris-HCl (pH 8.0) containing 20% sucrose, 5 mM EDTA, polymyxin B (100 μg/ml; Sigma-Aldrich), and lysozyme (0.5 mg/ml; Sigma-Aldrich) to release the periplasm content. The supernatants were harvested after 30 min of incubation at 4°C and precipitated by 60% ammonium sulfate saturation. The precipitate was dissolved in 10 mM Tris-HCl (pH 8.0) containing 0.3 M NaCl and filter sterilized prior to gel filtration on a Sephacryl-100 column (Amersham Pharmacia Biotech), followed by chromatography with an anion-exchange Mono Q column (Amersham Pharmacia Biotech). The LT-IIb preparations were analyzed for endotoxin content with the *Limulus* amoebocyte lysate assay kit (BioWhittaker, Inc., Walkersville, MD) using an *E. coli* K235 lipopolysaccharide standard.

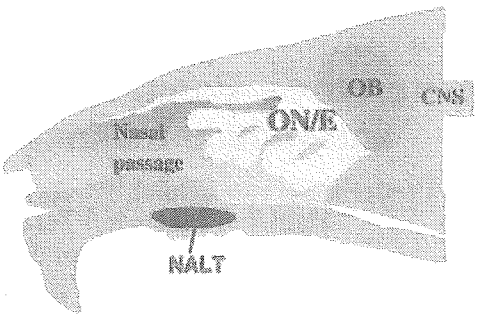

FIG. 1. Anatomy of the murine nasal tract and CNS. Indicated are the locations of the the ON/E, the NALT, and the OB in the nasal tract and adjacent CNS.

Radioiodination of proteins. Tetanus toxoid (TT) (kindly supplied by the Biken Institute, Osaka, Japan) was radiolabeled with ^{125}I. The radioiodination was performed with iodobeads (Pierce Chemicals) for 10 to 12 min at room temperature as described previously (46). Free, unincorporated ^{125}I was removed by dialysis using a Slide Dialyzer (Pierce Chemicals). The trichloroacetic acid-precipitable fraction of ^{125}I-labeled TT was used for all experiments described here. The specific activities of the radiolabeled proteins were 24.5 to 65 cpm/ng. A bicinchoninic acid protein assay (Pierce Chemicals) was used to determine the concentrations of radiolabeled proteins.

Nasal immunization. To assess the ability of ^{125}I-TT to target the ON/E following nasal application, a total of 20 μg of TT (~0.5×10^6 to 1.3×10^6 cpm) was administered in a 10-μl volume, i.e., 5 μl per nare, to naive mice. A total amount of 20 μg of ^{125}I-TT was given either alone or with the indicated enterotoxin delivered in the same volume as antigen alone. For the enterotoxins, we used 1 μg nCT, 10 μg mCT, and 10 μg mLTh-1, and for LTh-1, various amounts of protein were used, i.e., between 1 and 10 μg. For nasal application of the LTII-b enterotoxin, we used 5 μg of protein with ^{125}I-TT.

Trafficking of radiolabeled TT. We used radiolabeled TT protein to track its presence in both lymphoid and central nervous system (CNS) tissues. In these studies, ^{125}I-labeled-TT was given nasally. At 3, 6, 12, 24, and 48 h and 6 days, the ^{125}I-TT levels present in various lymphoid and CNS tissues were determined. For lymphoid tissues, the nasopharyngeal-associated lymphoreticular tissues (NALT), the cervical lymph nodes (CLNs), the mesenteric lymph nodes, the spleen, and blood (50 μl) were assessed. The isolation of NALT was performed as previously reported (49). For the CNS, we examined the ON/E, the olfactory bulbs (OB), and the remainder of the brain. These tissues were isolated as previously described (46). The radiolabeled TT in each tissue was quantitated by use of a gamma counter. The different nasal tract tissues isolated in this study are illustrated in Fig. 1.

In order to assess the influence of blocking the GM1 binding site of LTh-1 or nCT with subsequent tissue distribution of coadministered ^{125}I-TT after nasal application, the LTh-I and nCT were preincubated with a 15-fold molar excess of GM1 (Sigma-Aldrich) for 30 min at 25°C prior to nasal application. The cpm associated with different tissues 12 h after application were analyzed and compared with application without preincubation with GM1. A total of 20 μg of ^{125}I-TT and 5 μg of LTh-1 or 1 μg nCT either with or without preincubation with free GM1 was nasally administered to individual mice.

Sample collection. Blood was collected into heparinized collection tubes by retro-orbital bleeding of anesthetized mice. The plasma was separated from the cells by a 10-min centrifugation step at 10,000 × *g*. Nasal washes were collected by intubation of the trachea to access the nasopharyngeal cavity. This approach was used to avoid any blood contamination of the nasal washes. A total of 200 μl of phosphate-buffered saline (PBS) was inserted into the nasal cavity, and the

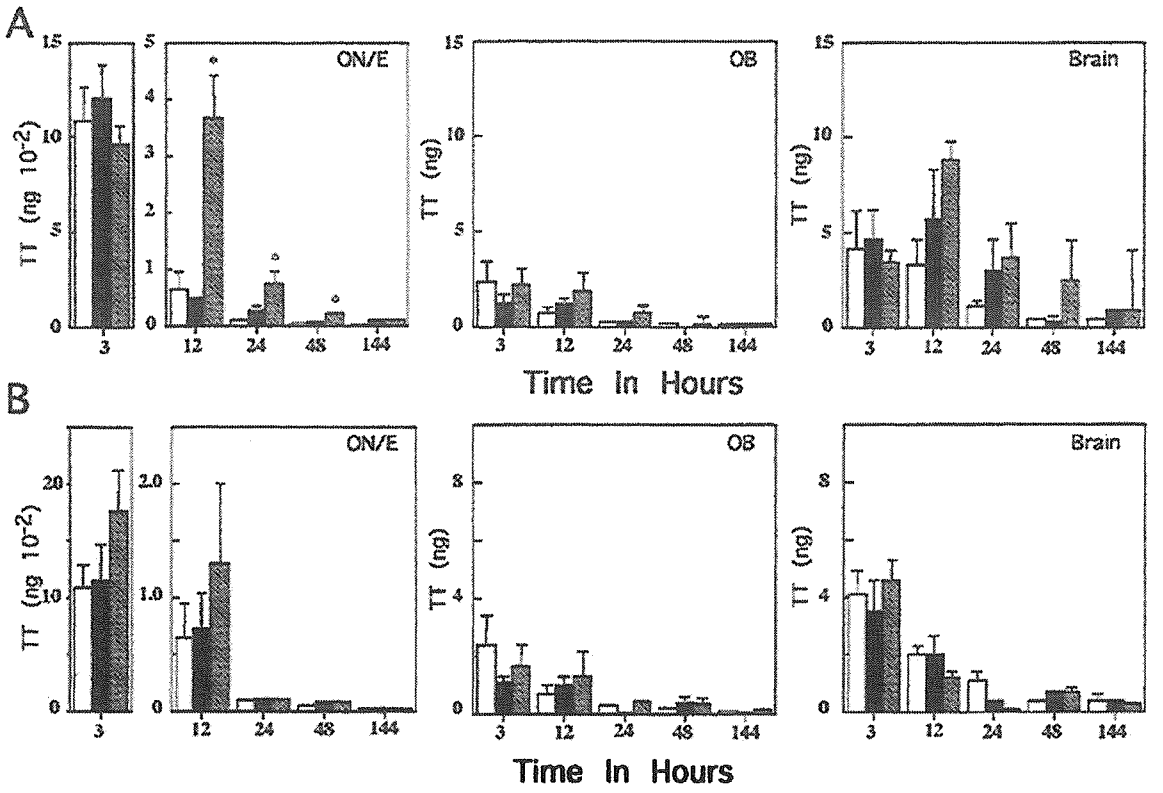

FIG. 2. Comparison of nCT- and mCT- (A) with nLTh-1- and mLTh-1-induced uptake (B) of ^{125}I-TT into olfactory and neuronal tissues. (A) Distribution of ^{125}I-TT in the ON/E, OB, and brain after nasal application of ^{125}I-TT alone (open bars) or in combination with 10 μg of mCT (solid bars) or 1.0 μg nCT (hatched bars) expressed as ^{125}I-TT protein (ng) accumulation. (B) Nasal application of ^{125}I-TT alone (open bars) or in combination with 10 μg of mLTh-1 (solid bars) or 1.0 μg of nLTh-1 (hatched bars) expressed as ^{125}I-TT protein (ng) accumulation. A total of 20 μg of ^{125}I-TT in 12 μl was given nasally either with or without enterotoxin (6 μl/nare). Significant differences between the ^{125}I-TT-only group and ^{125}I-TT-plus-enterotoxin group are indicated by an asterisk and mark *P* values smaller than 0.05. The averages of 4 to 10 mice plus 1 standard error of the mean are depicted.

exudate from the nares was collected in microcentrifuge tubes. Cells and debris were removed by a 10-min 10,000 × *g* centrifugation step. All samples were frozen at −80°C until they were analyzed by enzyme-linked immunosorbent assay (ELISA). Lymphoid and neuronal tissues were isolated as described previously (46).

Cytokine ELISA. The detection of the cytokines IL-6, IL-1β, and tumor necrosis factor alpha (TNF-α) in plasma and nasal washes was performed on Maxisorp 96-well immunoplates (Nunc A/S, Roskilde, Denmark). The plates were coated overnight at 4°C with the following capture monoclonal antibodies: rat anti-mouse IL-6 (clone MP5-20F3; BD PharMingen, San Diego, CA), rat anti-mouse IL-1β (clone 30311.1; R&D systems, Minneapolis, MN), and hamster anti-mouse TNF-α (clone TN3-19.12; BD PharMingen) at 2 μg/ml. The plates were washed with PBS-Tween 20 (0.05%) and blocked with 1% bovine serum albumin in PBS-Tween 20 (0.05%) for 1 hour at room temperature. Subsequently, the samples were added to 96-well plates and incubated overnight at 4°C. The plates were washed with PBS-Tween 20, and the biotinylated capture monoclonal antibodies rat anti-mouse IL-6 (clone MP5-32C11; BD PharMingen), goat anti-mouse IL-1β (R&D Systems), and rabbit anti-mouse TNF-α (BD PharMingen) at concentrations of 0.5 μg/ml, 300 ng/ml, and 0.5 μg/ml, respectively. For detection of IL-1β and TNF-α, streptavidin-conjugated to horseradish peroxidase (Life Technologies Inc., Rockville, MD) was used at a 1:2,000 dilution and anti-biotin-horseradish peroxidase at a 1:2,000 dilution (Vector Laboratories, Burlingame, CA) was used for IL-6. The ELISA plates were washed, followed by a 15-min incubation with 2,2'-azino-bis-(3)-ethylbenzylthiazoline-6-sulfonic acid substrate (Sigma Chemical Co., St. Louis, MO). The absorption at 415 nm was measured at various sample dilutions, and the cytokine levels were determined using standard curves. The detection limits of the ELISA for IL-6, IL-1β, and TNF-α were 10, 1, and 12 pg/ml, respectively.

Statistics. The data are expressed as the mean plus 1 standard error of the mean, and the results were compared by the two-tailed, unpaired Mann-Whitney or Student *t* test. The results were analyzed using the Statview II statistical program (Abacus Concepts, Berkeley, CA) adapted for MacIntosh computers.

RESULTS

Redirection of TT into the ON/E. In order to define the parameters involved in redirection of vaccine proteins into olfactory tissues by nCT and nLTh-1, the role of ADP-ribosyltransferase activity in antigen redistribution was first analyzed by comparison with nonenterotoxigenic mutants of CT (E112K) and LTh-1 (E112K). The presence of ^{125}I-TT in the ON/E, OB, and brain was assessed after nasal application of ^{125}I-TT only or in combination with nCT or mCT (E112K) (Fig. 2A) or with nLTh-1 or mLTh-1 (E112K) (Fig. 2B). The enterotoxin dose was based on the amount required to induce strong mucosal immune responses after nasal application. No significant differences were observed between the distribution of TT protein when given alone or with nCT (1 μg), mCT (10 μg), nLTh-1 (1 μg), or mLTh-1 (10 μg) in all tissues tested, with the exception of the ON/E. Strikingly elevated levels of TT protein were present at 12, 24, and 48 h in the ON/E (P = 0.004, P = 0.028, and P = 0.043, respectively) when given

nasally with nCT. However, minimal uptake was seen at these time points when TT was given alone or with mCT, nLTh-1 (1 μg), or mLTh-1 (Fig. 2A and B). Although the elevated redistribution of ^{125}I-TT into the ON/E induced by nCT was reproducible, it is unlikely that the 48-h time point was of biological importance, since the differences between the groups were relatively small. The nCT-versus-mCT results clearly show that ADP-ribosyltransferase activity is required for redirection of TT into the ON/E. However, it was noteworthy that nLTh-1 (1.0 μg) did not induce significant increases of TT protein in the ON/E at 12 h following nasal application. This TT accumulation was considerably lower than that seen with 1.0 μg of nCT and indicated that factors in addition to ADP-ribosyltransferase activity also play roles in protein redistribution. It should be noted that no preferential accumulation of TT was observed in the OB versus the brain as previously reported for CT-B and CT (46). Thus, unlike CT or CT-B, limited or no axonal transport of TT along olfactory neurons takes place after nasal application, and the distribution of TT in the OB and brain can be explained by the sizes of the organs and the blood associated with them.

Since nCT and nLTh-1 display different ganglioside binding specificities, where nLTh-1 binds in addition to GM1, as reported for nCT and also GD1b, GM2, asialo-GM1, and other intestinal glycoproteins (12, 18, 25), it remained possible that GM1 binding by the enterotoxin was required for protein redirection into the ON/E. Thus, decreased binding by nLTh-1 to GM1 may be due to competition by other nLTh-1 receptors in the nasal tract, which would inhibit protein redirection. In addition, it should be noted that the uptake of TT into various tissues, when 1 μg nLTh-1 was used, was about half of the total uptake observed when 1 μg of nCT was given nasally. To test the GM1 dependence of TT redirection, a dose-response experiment with nLTh-1 was performed (Fig. 3A). Increasing levels of nLTh-1 induced enhanced TT redirection, and this required between a two- and fivefold-higher dose to induce levels not significantly different from those seen with 1 μg of nCT. This observation is consistent with a requirement to target GM1 in order to redirect protein into the ON/E. A comparison between the abilities of nCT, mCT, nLTh-1, and mLTh-1 to redirect protein into ON/E was made (Fig. 3B). In conclusion, nCT was more potent than nLTh-1 in redirecting codelivered protein into the ON/E, while the nontoxic mCT or mLTh-1 was only marginally able to do so at the levels tested.

Distribution of TT in lymphoid tissues. After nasal application of ^{125}I-TT, the NALT, blood, spleen, and CLNs were isolated and analyzed for the distribution of protein when given alone or in combination with nCT, nLTh-1, mCT (E112K), and mLTh-1 (E112K). No significant differences were seen in these lymphoid tissues with the exception of NALT. A significantly decreased accumulation of TT was seen in NALT of mice given TT nasally with nCT (Fig. 4). A small decrease in TT protein accumulation in NALT was observed with mCT, and no significant differences were seen between nLTh-1 and mLTh-1 compared with TT given alone. The data from these observations are summarized in Table 1 and were compared with the observed immune responses induced by these enterotoxin-based mucosal adjuvants. It was interesting that only decreased antigen accumulation in NALT was observed when a strong Th2 response was induced.

The role of gangliosides in protein redirection. In order to assess the role of GM1 ganglioside binding by nLTh-1 and nCT for ^{125}I-TT redirection into the ON/E, the ganglioside binding site was blocked by prior incubation with a 15-fold molar excess of GM1. Blocking the ganglioside binding site of nLTh-1 and nCT with GM1 significantly inhibited redirection of ^{125}I-TT into the ON/E for both 5 μg of nLTh-1 ($P = 0.04$) and 1.0 μg of nCT ($P = 0.02$) 12 h after application (Fig. 5). Blocking the ganglioside binding site also elevated TT levels in the blood, spleen, and CLNs. A significant increase in TT accumulation was observed in the CLNs (which drain the nasal tract) 12 h after application with nLTh-1 (data not shown). Whether this increase of protein in the CLNs was due to a lack of ON/E targeting and resulted in subsequent drainage into the CLNs or was due to enhanced circulation in the blood, or a combination of the two, cannot be distinguished.

In order to determine whether binding to gangliosides other than GM1 would prevent antigen redirection into the ON/E, the heat-labile enterotoxin from serogroup two, LT-IIb, was used. This enterotoxin displays high-affinity binding to GD1a and GT1b and weak affinity for GM3 and does not bind at all to GM1 (12). Furthermore, LT-IIb is more toxic to Y1 adrenal cells than nCT based upon morphological changes and adenylate cyclase activation (24). When the ability of the LT-IIb enterotoxin was compared with that of nLTh-I for redirection of TT into the ON/E, it was very apparent that 5 μg of LT-IIb, unlike nLTh-I, was unable to redirect ^{125}I-TT into the ON/E and did not affect the TT distribution pattern observed in other tissues (Fig. 6). Thus, GM1 targeting appears to be an essential step in directing the ADP-ribosyltranferase to cause codelivered antigen redirection. As pointed out in Fig. 2, the TT associated with the OB was considerably lower than that observed in the brain and reflected the smaller size and lower amount of blood associated with these organs and argues against considerable axonal transport of TT from the nasal tract into the OB as observed with labeled CT and CT-B (46).

Differential production of inflammatory cytokines in the nasal tract. To determine if nasal application of mucosal adjuvants induces inflammatory cytokines, nasal washes and plasma were collected at various time points after nasal delivery. The nasal washes and plasma were analyzed for IL-1β, IL-6, and TNF-α. Differential expression of IL-6 was seen in the nasal washes (Fig. 7). Both nCT- and mCT-treated mice displayed IL-6 levels significantly elevated over those seen when TT was given alone (Fig. 7). Although the levels of IL-6 at 6 h were twofold higher in the nCT- than in the mCT-treated mice, no significant differences were seen between these groups until 12 h after application ($P = 0.026$). Markedly lower levels of IL-6 and IL-1β were seen in the plasma of the mice. The time frame between 3 and 12 h following administration of nCT and TT, when redirection of TT into the ON/E was observed (Fig. 2A), also represented the time when maximal IL-6 secretion was noted in nasal washes. Thus, local inflammatory responses were induced by nCT, and to a much lesser extent by mCT, during this time period. No detectable levels of TNF-α were observed in either plasma or nasal washes (data not shown), and IL-1β levels did not differ significantly among the three groups. These differences in production of IL-6 were not due to the differences in lipopolysaccharide, since the nCT contained ≤0.048 ng/μg and the mCT contained ≤1.0 ng/10

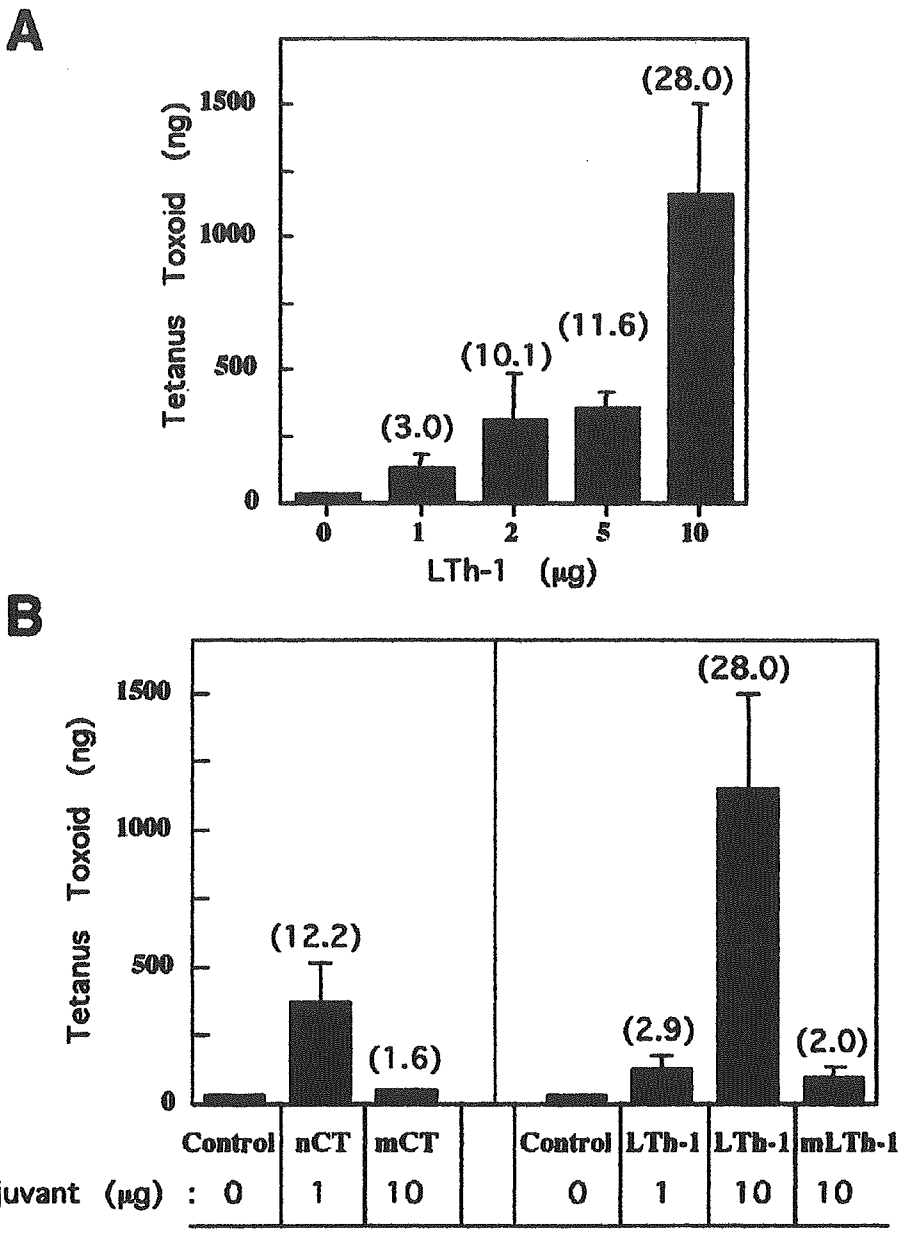

FIG. 3. Enterotoxin-dependent redirection of ^{125}I-TT into the ON/E. Various doses of nLTh-1, i.e., 0, 1, 2, 5, and 10 μg, were combined with 20 μg ^{125}I-TT and applied nasally. The ON/E were collected 12 h after application to assess redirection of the protein. (A) Indicated is the increase (*n*-fold) over control values, i.e., ^{125}I-TT alone, when administered with nLTh-I. (B) Comparison of the degrees of redirection of ^{125}I-TT into ON/E observed with nCT, mCT (E112K), nLTh-1, and mLTh-1 (E112K). Indicated are the means plus standard errors of the mean.

μg. The observations for IL-6 were confirmed by real-time reverse transcription-PCR on RNA derived from the ON/E (data not shown).

To determine whether LTh-1 had similar effects on IL-1β and IL-6 production, nasal washes and plasma were collected from mice treated 12 h prior with TT alone or TT with 1.0 or 5.0 μg of LTh-1. A significant increase in IL-6 was seen in nasal washes when 5 μg of LTh-1 was given with TT, while no significant increase was observed in plasma (Fig. 8). Elevated secretion of IL-6 was also seen in nasal washes with the 1.0-μg LTh-1 dose; however, this increase in IL-6 was not significant compared with TT alone.

DISCUSSION

The redirection of TT protein by native enterotoxin-based mucosal adjuvants raises questions regarding both safety and the molecular mechanisms involved. In this study, we addressed the parameters influencing redirection of the vaccine protein TT into the olfactory nerve/epithelium, NALT, and related lymphoid tissues, as well as the associated production of inflammatory cytokines in the nasal tract. To redirect nasally coadministered ^{125}I-TT into the ON/E by enterotoxin-based mucosal adjuvants, ADP-ribosyltransferase activity is clearly required. This is based upon the finding that both nCT and

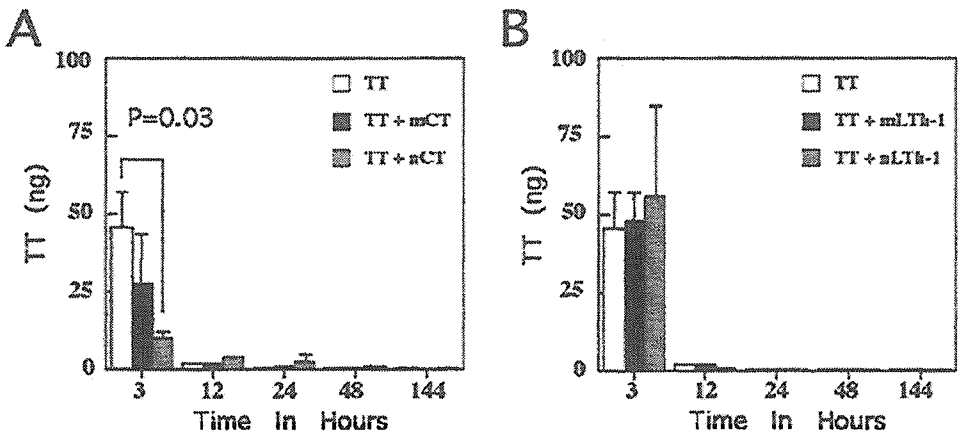

FIG. 4. Comparison of nCT- and nLTh-1- with mCT (E112K)- and mLTh-1 (E112K)-induced uptake of ^{125}I-TT into NALT. Distribution of ^{125}I-TT in NALT is expressed as TT (ng) accumulation per organ. A total of 20 μg of ^{125}I-TT alone or in combination with 10 μg of mCT or 1.0 μg of nCT (A) or ^{125}I-TT alone or in combination with 10 μg of mLTh-1 or 1.0 μg nLTh-1 (B) was given nasally (6 μl/nare). Accumulation of ^{125}I-TT was analyzed at various time points after application. The average of 5 to 10 mice plus standard error of the mean per data point are depicted.

nLTh-1 redirected protein into the ON/E while mCT (E112K) and mLTh-1 (E112K), which both lack ADP-ribosyltransferase activity (28, 54), did not. Thus, ADP-ribosyltransferase activity of the A subunit is an essential, although not sufficient, element for redirection of protein into the ON/E by AB_5 enterotoxins. Binding to GM1 by the B subunit, in addition to ADP-ribosyltransferase activity of the A subunit, also appears to be a prerequisite for redirection of protein into the ON/E, since incubation of nCT or nLTh-1 with excess GM1 prior to administration prevented accumulation of ^{125}I-TT in the ON/E.

The finding that LT-IIb does not redirect TT into the ON/E while it retains full ADP-ribosyltransferase activity and functions as a mucosal adjuvant when delivered nasally (34) may be explained by a requirement for GM1 binding by the enterotoxin to induce protein redirection, since LT-IIb, unlike nCT or nLTh-1, does not bind to GM1 gangliosides (12, 38). Using a human intestinal epithelial cell line (T84), others found that both nCT and LT-IIb bound with high affinity (2 to 5 μM) to the apical membranes of T84 cells (48). However, only nCT was able to elicit a cyclic-AMP-dependent secretory response. Moreover, while nCT-GM1 fractionated with a caveola-like, detergent-insoluble membrane fraction, the LT-IIb-GD1a complex was solubilized by 1% Triton X-100. The authors suggested that signal transduction may require the formation of caveola-like structures and demonstrated that the chimera composed of the LT-IIb A subunit and CT-B was capable of inducing a secretory response. Native CT binding to polarized epithelial cells takes place on the apical membrane surface but targets a basolaterally located effector molecule, i.e., adenylate cyclase (31). It could be hypothesized that the requirement to bind GM1 is associated with the endocytotic pathway taken following GM1 endocytosis. The LT-IIb binds to GD1a, a ganglioside that is not located in the caveola-like membrane domains as reported for GM1 (48), and may thus follow a different intracellular path in epithelial cells. As a consequence of this, the A1 subunits or the ADP-ribose-Gsα may not reach the adenylate cyclase located in the basolateral domain of polarized epithelial cells. This could be an important step for enhanced permeability of the epithelium and the ability of antigen to cross the nasal epithelial barrier.

The nasal tract is covered by a pseudostratified epithelium. Underneath this epithelium, a dense network of fenestrated capillaries provides a readily available blood supply (14). The nasal administration of enterotoxin-based adjuvants would target this epithelium through GM1 ganglioside binding. Our observations and those of others reporting the use of rabbit mucosa in vitro (14) clearly raise questions regarding the safety of nasal use of these adjuvants in humans. Human studies performed previously using nasal nLTh-1 and CT-B suggest that precautions need to be taken when applying these mole-

TABLE 1. Antigen-specific immune response and antigen distribution in NALT following nasal immunization

Adjuvant	Antigen	Plasma IgG[a]	S-IgA[b]	T helper activity	TT in NALT[c]	Reference(s)
	TT	+[d]	+	Th1/Th2	++++	30, 47, 51
nCT	TT	++++	+++	Th2	+	47, 50, 51
mCT(E112K)	TT	++++	+++	Th2	++	50, 51
nLTh-1	TT	++++	+++	Th1/Th2	++++	2, 7, 42
mLTh-1(E112K)	TT	++++	+++	Th1/Th2	++++	2, 7
LTIIb	AgI/II[e]	++++	+++	Th1/Th2	++++	34

[a] IgG, immunoglobulin G.
[b] S-IgA, mucosally-derived secretory IgA.
[c] Data were obtained in this study and indicate the relative TT antigen accumulation in NALT.
[d] +, low-, ++, medium-; +++, high-; ++++, very high.
[e] AgI/II, Antigen I/II from *Streptococcus mutans*.

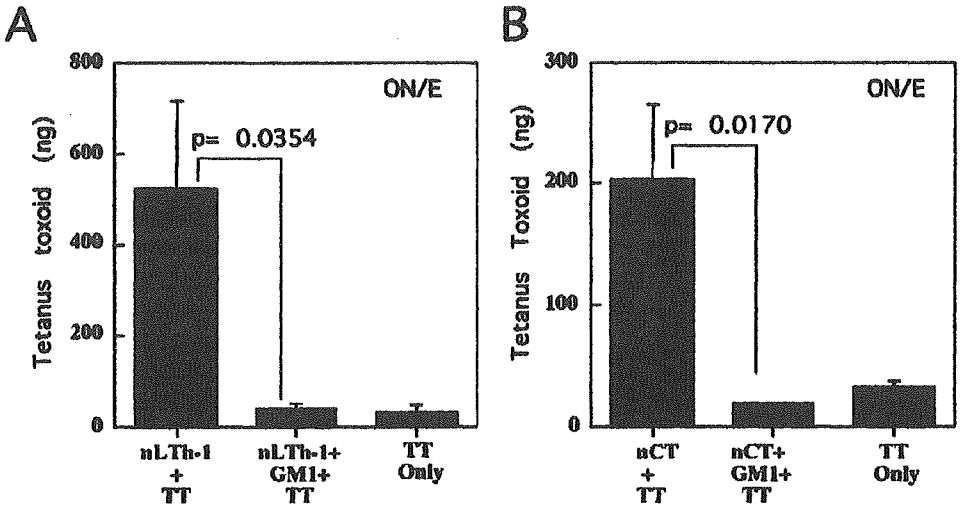

FIG. 5. Influence of blocking the GM1 binding site on nLTh-1 and nCT on tissue distribution of coadministered ^{125}I-TT in the ON/E after nasal application. nLTh-I (A) and nCT (B) were preincubated with a 15-fold molar excess of GM1 for 30 min at room temperature prior to nasal application together with ^{125}I-TT. The cpm associated with the ON/E 12 h after application were analyzed and compared with application without preincubation with GM1 and with administration of ^{125}I-TT antigen alone. A total of 20 μg ^{125}I-TT with 5 μg of nLTh-1 or 1.0 μg of nCT was nasally delivered to individual mice. The results are from five mice per group. Indicated are the means plus standard errors of the mean.

cules to the human nasal tract. Human studies involving nasal application of CT-B reported mild adverse effects, which resolved within 24 h (1, 39–41). The tolerated and effective dose was between 100 and 500 μg for nasally applied CT-B in a nasal spray/aerosol. At the lower end of an effective immunization range, i.e., 100 μg of CT-B, 7 out of 20 patients and all in the high range (the 1,000-μg group), experienced adverse effects. The symptoms consisted of profuse nasal secretions, itching, and violent sneezing, which resolved within 1 day (1). Although the use of a high-dose CT-B is different from our toxin-mediated antigen redirection, it could be argued that high doses of CT-B will accumulate in the olfactory neuroepithelium, as has been reported for mice (46), and consequently would lead to induction of proinflammatory cytokines throughout the nasal tract.

The results with nasally applied CT-B in humans are consistent with our observations that high levels of IL-6, and to a lesser extent IL-1β, are present in the nasal tract during the first 24 h after application. Interleukin 6 was expressed during the peak of TT protein redirection into the ON/E, and at 12 h was significantly higher in nCT-treated mice than in mice treated with mCT E112K. Furthermore, both enterotoxins induced significantly higher IL-6 levels in nasal washes than were seen in mice given TT only. IL-6 is a multifunctional cytokine that influences both innate immune reactions, such as inflammation (36) and acute-phase responses, and specific immunity, such as B-cell differentiation. IL-6 is produced by a variety of cells, including epithelial cells, macrophages, fibroblasts, and T cells. Native CT rapidly induces IL-6 secretion by the rat intestinal epithelial cell line IEC-6 (35). Whether IL-6 plays a

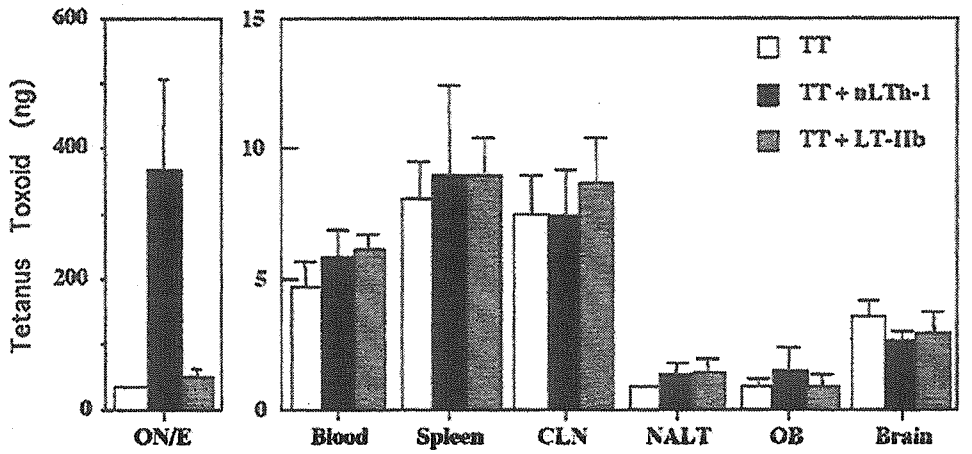

FIG. 6. Trafficking of ^{125}I-TT (20 μg) given nasally without or with nLTh-1 (5 μg) or LT-IIb (5 μg) as mucosal adjuvant. The uptake of ^{125}I-TT into the ON/E, OB, brain, NALT, CLNs, blood, and spleen is shown 12 h after nasal application. The results depicted are from five mice/group and are representative of three separate experiments. Indicated are the means plus standard errors of the mean.

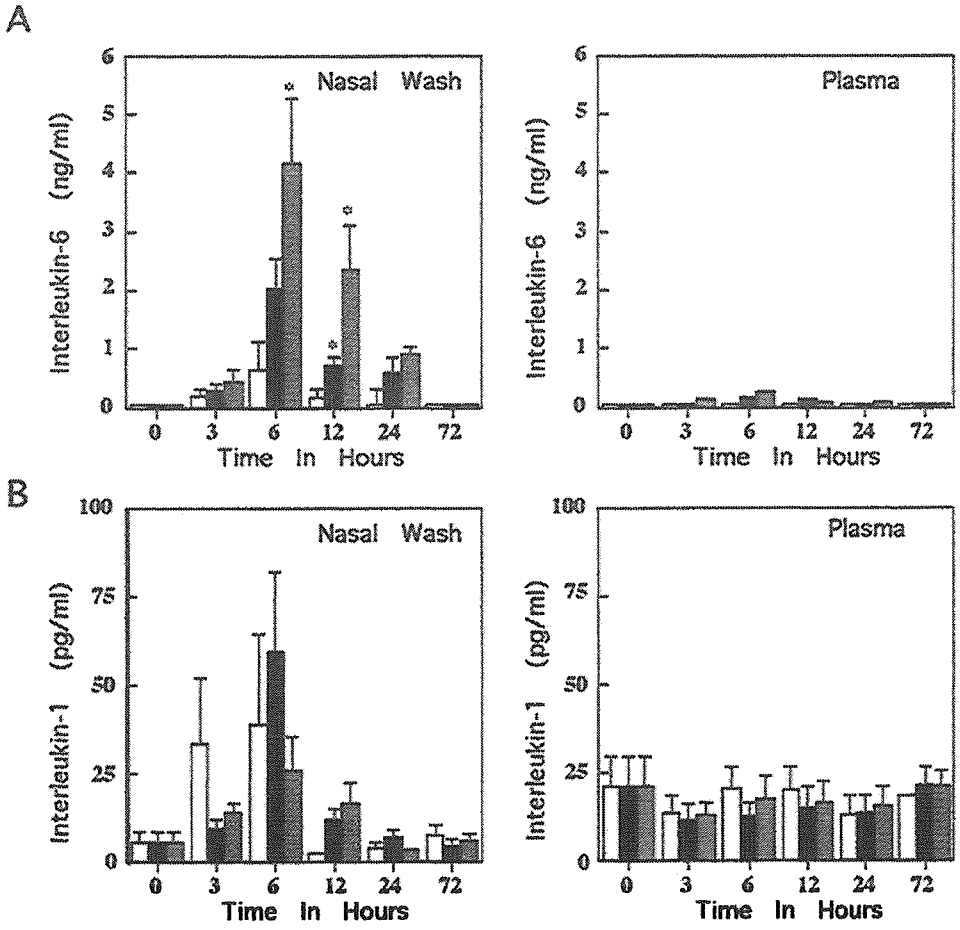

FIG. 7. Inflammatory cytokine expression in the nasal tract after nasal application of TT with or without mCT or nCT. Nasal washes were collected at 0, 3, 6, 12, and 24 h after nasal application of TT alone (open bars), TT and mCT (solid bars), or TT and nCT (hatched bars). The inflammatory cytokine levels for IL-6 (A), IL-1β (B), and TNF-α were determined by ELISA. No TNF-α was detected in the nasal washes. Indicated are the means plus standard errors of the mean for IL-6 and IL-1β. The asterisks indicate significantly elevated cytokine levels ($P < 0.05$) when nCT or mCT with TT was compared with TT alone. The results are representative of two separate experiments.

role in antigen redirection remains to be determined; however, it is more likely that multiple factors contribute to antigen redirection into the ON/E. Specifically, neuropeptides could be major players in this process. For example, vasoactive intestinal peptide (VIP) plays an important role in fluid accumulation in the rat jejunum following stimulation with nCT or nLTh-1 (27). Furthermore, CT-B seems to specifically target VIP-containing neurons in the intestinal tract (15). These observations indicate that VIP could also be important for nasal reactogenicity and antigen redirection.

Human studies involving nasal application of CT-B have focused on the induced immune responses to CT-B rather than on its properties as a mucosal adjuvant (1, 39–41). However, the adjuvant properties of nLTh-1 were assessed in humans given two nasal applications a week apart with an aerosolized virosome-formulated influenza vaccine containing 1.0 μg or 2.0 μg of nLTh-1 for induction of influenza virus-specific immune responses (16, 17). The nLTh-1 functioned as a mucosal adjuvant in humans and induced influenza virus-specific immune responses; however, about 50% of the subjects experienced some type of local or systemic adverse reaction. These reactions included rhinorrhea, stuffiness, sneezing, and headaches, but most of them were mild and resolved within 48 h (16). Similar reactions have also been reported with nasal influenza virus vaccine given with 99.5 μg LT-B and 0.5 μg nLTh-1 (23). The results are consistent with our observations with nCT and nLTh-1 in that redirection of coadministered antigen into the ON/E and production of inflammatory cytokines resolved within 48 h, indicating that similar events could have taken place in humans.

Despite the similarities between mice and humans, the relative surfaces of the nasal tract that constitute the olfactory epithelium are quite different. In mice, approximately 45% of the nasal tract surface constitutes olfactory epithelium (19), while in humans it is an estimated 2.5 cm^2 which would translate to ~2 to 3% of the nasal surface (13, 22). Thus, in mice, nasal application is probably more likely to target olfactory neurons than in humans. Furthermore, the nasopharynx has a 90° angle in humans while there is only a 15° angle in mice. Due to the much larger volume of the human nasopharynx (20 ml) than the mouse nasopharynx (30 μl) and the larger angle, it is likely much harder to consistently target the olfactory

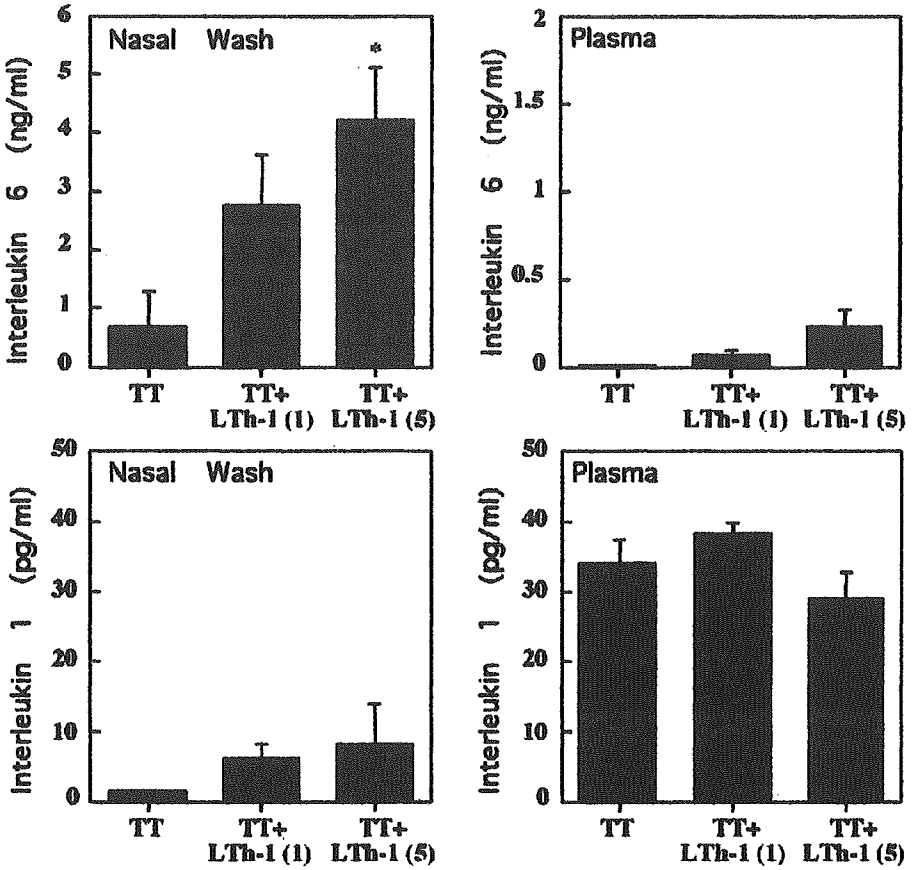

FIG. 8. Inflammatory cytokine expression in the nasal tract after nasal application of TT with or without LTh-1. Nasal washes and plasma were collected 12 h after nasal application of TT (10 μg) alone, TT and LTh-1 (1) (1.0 μg), or TT and LTh-1 (5) (5.0 μg). The levels of IL-6 and IL-1β in nasal washes and plasma were measured by ELISA. Indicated are the means plus standard errors of the mean for IL-6 and IL-1β of five mice per group. The asterisk indicates significantly elevated cytokine levels ($P < 0.05$) when LTh-1 given with TT was compared with TT given alone. The results are representative of three separate experiments.

epithelium in humans using nasal drops, while in mice this would be very reproducible (13). It could be argued that because of the above-outlined reasons nasal sprays would more consistently target the olfactory epithelium in humans than nasal drops.

The observation that nCT significantly reduces TT accumulation in NALT 3 h after nasal application compared to TT alone or TT plus nLTh-1 (Fig. 4) is interesting from the perspective that exposure to a low dose of soluble protein is associated with induction of a Th2-type T helper cell response (6, 20, 37, 47). The induction of potent Th2-type helper activity specific for antigens codelivered with nCT (33, 50) or mCT (30, 51, 53, 54) and the induction of a mixed Th1/Th2 response to antigen coadministered with nLTh-1 (2, 7, 42), mLTh-1 (2, 7), or LTIIb (34) coincide with decreased antigen accumulation in NALT with a strong Th2 response but not with the mixed Th1/Th2 response (Table 1). For example, antigen accumulation in NALT is approximately sixfold lower with nCT than with nLTh-1. It will be interesting to see in future studies whether this altered antigen level will translate into an altered cytokine environment in the NALT for induction of a TT-specific immune response.

In summary, the redirection of a vaccine protein into the olfactory tissues by enterotoxin-based mucosal adjuvants following nasal administration is associated with reactogenicity in the nasal mucosa. The differential accumulation of TT protein in NALT when administered with nCT or nLTh-1 may have consequences for the induced TT-specific T helper cell responses. The parameters controlling antigen redirection into the ON/E include ADP-ribosyltransferase activity of the A subunit and GM1 ganglioside binding by the B subunit. Thus, redirection of vaccine antigen into the ON/E by enterotoxin-based mucosal adjuvants, such as nCT and nLTh-1, clearly requires both ADP-ribosyltransferase activity and targeting of GM1 gangliosides.

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REFERENCES

1. Bergquist, C., E.-L. Johansson, T. Lagergard, J. Holmgren, and A. Rudin. 1997. Intranasal vaccination of humans with recombinant cholera toxin B subunit induces systemic and local antibody responses in the upper respiratory tract and vagina. Infect. Immun. 65:2676–2684.
2. Cheng, E., L. Cardenas-Freytag, and J. D. Clements. 1999. The role of

cAMP in mucosal adjuvanticity of *Escherichia coli* heat-labile enterotoxin (LT). Vaccine **18**:38–49.
3. **Chong, C., M. Friberg, and J. D. Clements.** 1998. LT(R192G), a non-toxic mutant of the heat-labile enterotoxin of *Escherichia coli*, elicits enhanced humoral and cellular immune responses associated with protection against lethal oral challenge with *Salmonella* spp. Vaccine **16**:732–740.
4. **Clements, J. D., N. M. Hartzog, and F. L. Lyon.** 1988. Adjuvant activity of *Escherichia coli* heat-labile enterotoxin and effect on the induction of oral tolerance in mice to unrelated protein antigens. Vaccine **6**:269–277.
5. **Connell, T. D., and R. K. Holmes.** 1992. Molecular genetic analysis of ganglioside GD1b-binding activity of *Escherichia coli* type IIa heat-labile enterotoxin by use of random and site-directed mutagenesis. Infect. Immun. **60**:63–70.
6. **Constant, S. L., and K. Bottomly.** 1997. Induction of Th1 and Th2 $CD4^+$ T cell responses: the alternative approaches. Annu. Rev. Immunol. **15**:297.
7. **de Haan, L., W. R. Verwey, I. K. Feil, M. Holtrop, W. G. J. Hol, E. Agsteribbe, and J. Wilschut.** 1998. Role of GM1 binding in the mucosal immunogenicity and adjuvant activity of the *Escherichia coli* heat-labile enterotoxin and its B subunit. Immunology **94**:424–430.
8. **Dickinson, B. L., and J. D. Clements.** 1995. Dissociation of *Escherichia coli* heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. Infect. Immun. **63**:1617–1623.
9. **Douce, G., C. Turcotte, I. Cropley, M. Roberts, M. Pizza, M. Domenghini, R. Rappuoli, and G. Dougan.** 1995. Mutants of *Escherichia coli* heat-labile toxin lacking ADP-ribosyltransferase activity act as nontoxic mucosal adjuvant. Proc. Natl. Acad. Sci. USA **92**:1644–1648.
10. **Elson, C. O., and W. Ealding.** 1984. Generalized systemic and mucosal immunity in mice after mucosal stimulation with cholera toxin. J. Immunol. **132**:2736–2741.
11. **Elson, C. O., and W. Ealding.** 1985. Genetic control of the murine immune response to cholera toxin. J. Immunol. **135**:930–932.
12. **Fukuta, S., J. L. Magnani, E. M. Twiddy, R. K. Holmes, and V. Ginsburg.** 1988. Comparison of the carbohydrate-binding specificities of cholera toxin and *Escherichia coli* heat-labile enterotoxins LTh-I, LT-IIa, and LT-IIb. Infect. Immun. **56**:1748–1753.
13. **Gizurarson, S.** 1990. Animal models for intranasal drug delivery studies. Acta Pharm. Nord. **2**:105–122.
14. **Gizurarson, S., S.-I. Tamura, T. Kurata, K. Hasiguchi, and H. Ogawa.** 1991. The effect of cholera toxin and cholera toxin B subunit on the nasal mucosal membrane. Vaccine **9**:825–832.
15. **Gizurarson, S., S. Tamura, C. Aizawa, and T. Kurata.** 1992. Stimulation of the transepithelial flux of influenza HA vaccine by cholera toxin B subunit. Vaccine **19**:101–106.
16. **Gluck, R., R. Mischler, P. Durrer, E. Furer, A. B. Lang, C. Herzog, and S. J. Cryz, Jr.** 2000. Safety and immunogenicity of intranasally administered inactivated trivalent virosome-formulated influenza vaccine containing *Escherichia coli* heat-labile toxin as a mucosal adjuvant. J. Infect. Dis. **181**:1129–1132.
17. **Gluck, U., J.-O. Gebbers, and R. Gluck.** 1999. Phase 1 evaluation of intranasal virosomal influenza vaccine with and without *Escherichia coli* heat-labile toxin in adult volunteers. J. Virol. **73**:7780–7786.
18. **Griffiths, S. L., R. A. Finkelstein, and R. R. Critchley.** 1986. Characterization of the receptor for cholera toxin and *Escherichia coli* heat-labile enterotoxin in rabbit intestinal brush borders. Biochem. J. **238**:313–322.
19. **Gross, E. A., J. A. Swenberg, S. Fields, and J. A. Popp.** 1982. Comparative morphometry of the nasal cavity in rats and mice. J. Anat. **135**:83–88.
20. **Guery, J.-C., F. Galbiati, S. Smiroldo, and L. Adorini,.** 1996. Selective development of T helper (Th) 2 cells induced by continuous administration of low dose soluble proteins to normal and β2-microglobulin-deficient BALB/c mice. J. Exp. Med. **183**:485–497.
21. **Guidry, J. J., L. Cardenas, E. Cheng, and J. D. Clements.** 1997. Role of receptor binding in toxicity, immunogenicity, and adjuvanticity of *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **65**:4943–4950.
22. **Harkema, J. R.** 1991. Comparative aspects of nasal airway anatomy: relevance to inhalation toxicology. Toxicol. Pathol. **19**:321–336.
23. **Hashigucci, K., H. Ogawa, T. Ishidate, R. Yamashita, H. Kamiya, K. Watanabe, N. Hattori, T. Sato, Y. Suzuki, T. Nagamine, C. Aizawa, S.-I. Tamura, T. Kurata, and A. Oya.** 1995. Antibody responses in volunteers induced by nasal influenza vaccine combined with *Escherichia coli* heat-labile enterotoxin B subunit containing a trace amount of holotoxin. Vaccine **14**:113–119.
24. **Holmes, R. K., E. M. Twiddy, and C. L. Pickett.** 1986. Purification and characterization of type II heat-labile enterotoxin of *Escherichia coli*. Infect. Immun. **53**:464–473.
25. **Holmgren, J., M. Lindblad, P. Fredman, L. Svennerholm, and H. Myrvold.** 1985. Comparison of receptors for cholera and *Escherichia coli* enterotoxins in human intestine. Gastroenterology **89**:27–35.
26. **Holmgren, J., P. Fredman, M. Lindblad, and L. Svennerholm.** 1982. Rabbit intestinal glycoprotein receptor for *Escherichia coli* heat-labile enterotoxin lacking affinity for cholera toxin. Infect. Immun. **38**:424–433.
27. **Jiang, M. M., A. Kirchgessner, M. D. Gershon, and A. Suprenant.** 1993. Cholera toxin-sensitive neurons in guinea pig submucosal plexus. Am. J. Physiol. **264**:G86.
28. **Komase, K., S.-I. Tamura, K. Matsuo, K. Watanabe, N. Hattori, A. Odaka, Y. Suzuki, T. Kurata, and C. Aizawa.** 1997. Mutants of *Escherichia coli* heat-labile enterotoxin as an adjuvant for nasal influenza vaccine. Vaccine **16**:248–254.
29. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154**:367–382.
30. **Kweon, M.-N., M. Yamamoto, F. Watanabe, S. Tamura, F. W. van Ginkel, A. Miyauchi, H. Takagi, Y. Takeda, T. Hamabata, K. Fujihashi, J. R. McGhee, and H. Kiyono.** 2002. A non-toxic chimeric enterotoxin adjuvant induces protective immunity in both mucosal and systemic compartments with reduced IgE antibodies. J. Infect. Dis. **186**:1261–1269.
31. **Lencer, W. I., J. B. de Almeida, S. Moe, J. L. Stow, D. A. Ausiello, and J. L. Madara.** 1993. Entry of cholera toxin into polarized human intestinal epithelial cells. J. Clin. Investig. **92**:2941–2951.
32. **Lycke, N., U. Karlsson, A. Sjolander, and K.-E. Magnusson.** 1991. The adjuvant action of cholera toxin is associated with an increased intestinal permeability for luminal antigen. Scand. J. Immunol. **33**:691–698.
33. **Marinaro, M., H. F. Staats, T. Hiroi, R. J. Jackson, M. Coste, P. N. Boyaka, N. Okahashi, M. Yamamoto, H. Kiyono, H. Bluethmann, K. Fujihashi, and J. R. McGhee.** 1995. Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4. J. Immunol. **155**:4621–4629.
34. **Martin, M., D. J. Metzger, S. M. Michalek, T. D. Connell, and M. W. Russell.** 2000. Comparative analysis of the mucosal adjuvanticity of the type II heat-labile enterotoxins LT-IIa and LT-IIb. Infect. Immun. **68**:281–287.
35. **McGee, D. W., C. O. Elson, and J. R. McGhee.** 1993. Enhancing effect of cholera toxin on interleukin-6 secretion by IEC-6 intestinal epithelial cells: mode of action and augmenting effect of inflammatory cytokines. Infect. Immun. **61**:4637–4644.
36. **Paul, R., U. Koedel, F. Winkler, B. C. Kieseier, A. Fontana, M. Kopf, H. P. Hartung, and H. W. Pfister.** 2003. Lack of IL-6 augments inflammatory response but decreases vascular permeability in bacterial meningitis. Brain **126**:1873–1882.
37. **Pfeiffer, C., K. Stein, S. H. K. Southwood, A. Sette, and K. Bottomly.** 1995. Altered peptide ligands can control CD4 T lymphocytes differentiation *in vivo*. J. Exp. Med. **181**:1569.
38. **Pickett, C. L., E. M. Twiddy, B. W. Belisle, and R. K. Holmes.** 1986. Cloning of genes that encode a new heat-labile enterotoxin of *Escherichia coli*. J. Bacteriol. **165**:348–352.
39. **Quiding-Jarbrink, M., I. Nordstrom, G. Granstrom, A. Kilander, M. Jertborn, E. C. Butcher, A. I. Lazarovits, J. Holmgren, and C. Czerkinsky.** 1997. Differential expression of tissue-specific adhesion molecules on human circulating antibody-forming cells after systemic, enteric, and nasal immunizations: a molecular basis for the compartmentalization of effector B cell responses. J. Clin. Investig. **99**:1281–1286.
40. **Rudin, A., E.-L. Johansson, C. Bergquist, and J. Holmgren.** 1998. Differential kinetics and distribution of antibodies in serum and nasal and vaginal secretions after nasal and oral vaccination of humans. Infect. Immun. **66**:3390–3396.
41. **Rudin, A., G. C. Rijse, and J. Holmgren.** 1999. Antibody responses in the lower respiratory tract and male urogenital tract in humans after nasal and oral vaccination with cholera toxin B subunit. Infect. Immun. **67**:2884–2890.
42. **Takahashi, I., M. Marinaro, H. Kiyono, R. J. Jackson, I. Nakagawa, K. Fujihashi, S. Hamada, J. D. Clements, K. L. Bost, and J. R. McGhee.** 1996. Mechanisms for mucosal immunogenicity and adjuvancy of *Escherichia coli* labile enterotoxin. J. Infect. Dis. **173**:627–635.
43. **Tsuji, T., T. Inoue, A. Miyama, and M. Noda.** 1991. Glutamic acid-112 of the A subunit of heat-labile enterotoxin from enterotoxigenic *Escherichia coli* is important for ADP-ribosyltransferase activity. FEBS Lett. **291**:319–321.
44. **Tsuji, T., T. Inoue, A. Miyama, K. Okamoto, T. Honda, and T. Miwatani.** 1990. A single amino acid substitution in the A subunit of *Escherichia coli* enterotoxin results in a loss of its toxic activity. J. Biol. Chem. **265**:22520–22525.
45. **Uesaka, Y., Y. Otsuka, Z. Lin, S. Yamasaki, J. Yamaoka, H. Kurazono, and Y. Takeda.** 1994. Simple method of purification of *Escherichia coli* heat-labile enterotoxin and cholera toxin using immobilized galactose. Microb. Pathog. **16**:71–76.
46. **van Ginkel, F. W., R. J. Jackson, Y. Yuki, and J. R. McGhee.** 2000. The mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. J. Immunol. **165**:4778–4782.
47. **Wang, L.-F., J.-Y. Lin, K.-H., Hsieh and R.-H. Lin.** 1996. Epicutaneous exposure of protein antigen induces a predominant Th2-like response with high IgE production in mice. J. Immunol. **156**:4079.
48. **Wolf, A., M. G. Jobling, S. Wimer-Mackin, M. Ferguson-Maltzman, J. L. Madara, R. K. Holmes, and W. L. Lencer.** 1998. Ganglioside structure dictates signal transduction by cholera toxin and association with caveolae-like membrane domains in polarized epithelia. J. Cell Biol. **141**:917–927.
49. **Wu, H.-Y., H. H. Nguyen, and M. W. Russell.** 1997. Nasal lymphoid tissue (NALT) as a mucosal immune inductive site. Scand. J. Immunol. **46**:506–513.
50. **Xu-Amano, J., H. Kiyono, R. J. Jackson, H. F. Staats, K. Fujihashi, P. D.**

Burrows, C. O. Elson, S. Pillai, and J. R. McGhee. 1993. Helper T cell subsets for immunoglobulin A response: oral immunization with tetanus toxoid and cholera toxin as adjuvant selectively induces Th2 cells in mucosa associated tissues. J. Exp. Med. **178**:1309–1320.

51. **Yamamoto, M., D. E. Briles, S. Yamamoto, M. Ohmura, H. Kiyono, and J. R. McGhee.** 1998. A nontoxic adjuvant for mucosal immunity to pneumococcal surface protein A. J. Immunol. **161**:4115–4121.
52. **Yamamoto, M., H. Kiyono, S. Yamamoto, E. Batanero, M.-N. Kweon, S. Otake, M. Azuma, Y. Takeda, and J. R. McGhee.** 1999. Direct effects on antigen-presenting cells and T lymphocytes explain the adjuvanticity of a nontoxic cholera toxin mutant. J. Immunol. **162**:7015–7021.
53. **Yamamoto, S., Y. Takeda, M. Yamamoto, H. Kurazono, K. Imaoka, K. Fujihashi, M. Noda, H. Kiyono, and J. R. McGhee.** 1997. Mutants in the ADP-ribosyltransferase cleft of cholera toxin lack diarrheagenicity but retain adjuvanticity. J. Exp. Med. **185**:1203–1210.
54. **Yamamoto, S., H. Kiyono, M. Yamamoto, K. Imaoka, K. Fujihashi, F. W. van Ginkel, M. Noda, Y. Takeda, and J. R. McGhee.** 1997. A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. Proc. Natl. Acad. Sci. USA **94**:5267–5272.
55. **Zemelman, B. V., S. H. Chu, and W. A. Walker.** 1989. Host response to *Escherichia coli* heat-labile enterotoxin via two microvillus membrane receptors in the rat intestine. Infect. Immun. **57**:2947–2952.

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