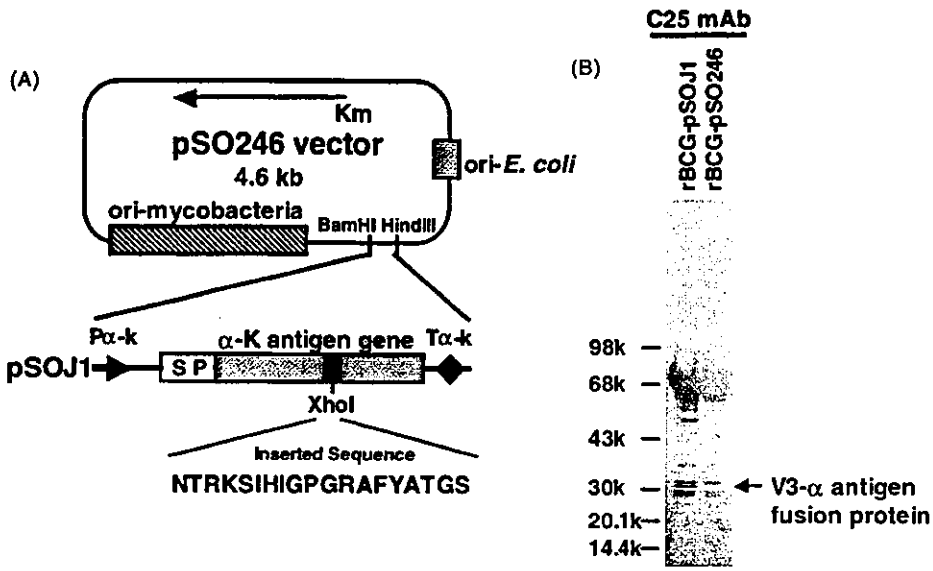

Fig. 1. Construction of expression vector and expression of the V3J1/α-antigen chimeric protein. (A) Construction of pSOV3J1 vector. A DNA fragment, which is optimized for mycobacterial codon usage, encoding the HIV-1 V3-PND region from Japanese consensus HIV-1 V3 were inserted into the *Xho* I site in the α-antigen gene in plasmid pSO246. (B) Detection of the V3J1/α-antigen chimeric protein in the culture supernatants by Western blotting.

HIV strain, yielding a recombinant BCG clone which was designated rBCG–pSOV3J1 (Fig. 1A). The rBCG secreted a chimeric protein of HIV-1 V3J1 and α-antigen which was detected in culture filtrates by Western blot as a 34 kDa molecule (Fig. 1B). The concentration of the secreted protein was determined to be 8.5±2.3 μg/ml, and secretion persisted for at least 450 passages in vitro.

3.2. Influence of inoculation route on immune induction with rBCG–pSOV3J1

rBCG–pSOV3J1 was then used to investigate the influence of various routes of inoculation on the immunogenicity of HIV-1V3J1. Forty guinea pigs were divided into five groups of eight animals each. The first group received a single i.d. inoculation of rBCG–pSOV3J1 (0.1 mg), the second group received four i.n. inoculations (0.01 mg each) once a week for four consecutive weeks, the third group received four i.r. inoculations (80 mg each) once a week for four consecutive weeks, the fourth group received three i.r. inoculations of rBCG–pSOV3J1 followed by a s.c. inoculation (1.0 mg) and the fifth group received three i.r. inoculations followed by an i.d. inoculation (0.1 mg). An additional 20 guinea pigs were inoculated with rBCG–pSO246 as a vector control (four animals per group) and five healthy guinea pigs were inoculated with PBS as naïve controls (one animal per group).

DTH responses to HIV-V3J1 peptide and PPD were analyzed in each of the guinea pigs immunized by various routes with rBCG–pSOV3J1 (Fig. 2). The DTH responses to PPD 7 weeks after the final inoculation were significantly higher in all the five groups of immunized guinea pigs compared to controls, with a mean diameter of induration ranging from 14.6 to 21.0 mm (dark columns in Fig. 2). In contrast, HIV-V3J1 peptide-specific responses were variable according to the route of inoculation (open columns in Fig. 2). Significantly higher DTH responses to HIV-V3J1 peptide were

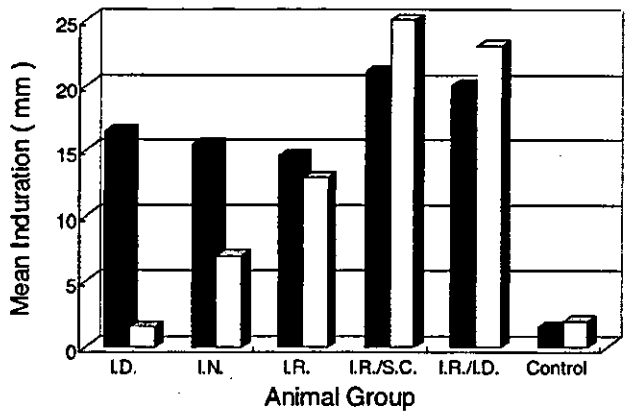

Fig. 2. Induction of DTH skin reactions against PPD and HIV-1 V3J1 in guinea pigs immunized with rBCG–pSOV3J1 via various routes. Forty guinea pigs were divided into five groups of eight animals each. The first group received a single i.d. inoculation (0.1 mg), the second group received four i.n. inoculations (0.01 mg each), the third group received four i.r. inoculations (80 mg each), the fourth group received three i.r. inoculations followed by a s.c. inoculation (1.0 mg) and the fifth group received three i.r. inoculations followed by an i.d. inoculation (0.1 mg). An additional five healthy guinea pigs were inoculated with PBS as naïve controls (one animal per group). The animals were injected dermally with PPD (0.5 μg) or KLH conjugate–V3J1 peptide (40 μg) 7 weeks after inoculation. Data indicate the mean diameter of induration 24 h after injection of the antigens.

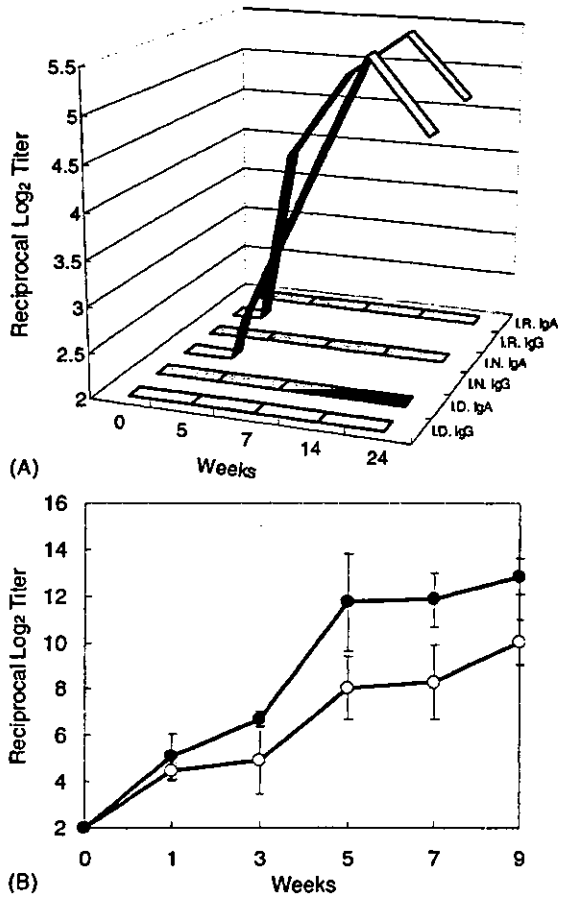

Fig. 3. Combined intrarectal and intradermal inoculation of rBCG–pSOV3J1 induces high levels of V3J1-specific humoral immunity in guinea pigs. (A) Induction of antibody responses to HIV-1 V3J1 by intradermal, nasal and rectal inoculations of rBCG–pSOV3J1. (B) Induction of significant levels of anti-V3J1 serum IgG by combined i.r./s.c. inoculations (closed circle) or i.r./i.d. inoculations (open circle). Data are shown as the mean antibody titer ± S.D.

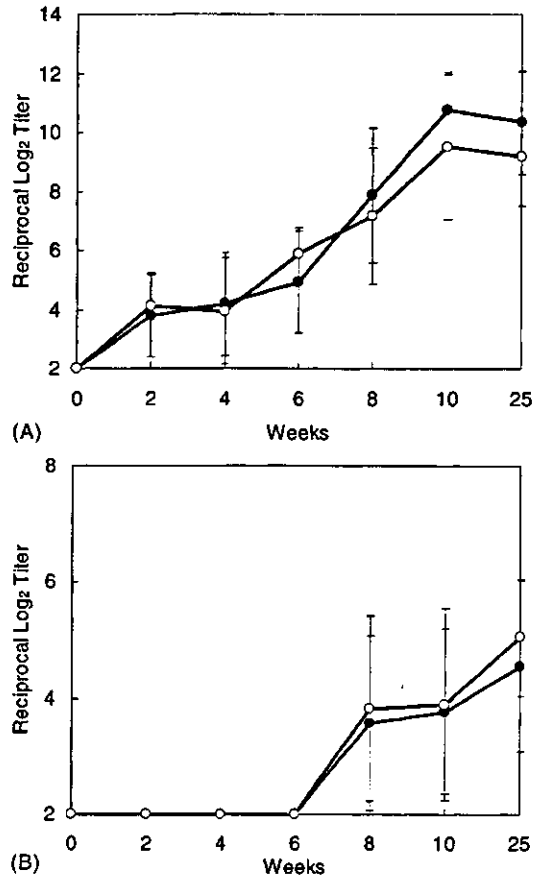

Fig. 4. Optimized inoculation of rBCG–pSOV3J1 induces high levels of V3J1-specific humoral immunity in guinea pigs. Twenty-four guinea pigs were divided into three groups of eight animals each. The first group (closed circle) received two i.r. inoculations (80 mg each) followed by a s.c. inoculation (1.0 mg), the second group (open circle) received two i.r. inoculations (80 mg each) followed by an i.d. inoculation (0.1 mg) and the third group of animals was inoculated with rBCG–pSO246 as a vector control. (A) Induction of V3J1-specific IgG responses in sera from the two immunized groups. (B) Induction of V3J1-specific IgA responses in sera of the same animals. Results represent the mean antibody titer ± S.D. The control group induced undetectable levels of serum antibody (data not shown).

observed in guinea pigs immunized by combined intrarectal and cutaneous routes (mean diameters of induration of 23.0 and 25.0 mm, respectively) compared with i.d., i.n., or i.r. inoculations (1.5, 6.0, and 12.9 mm diameters, respectively). No DTH responses were observed in control guinea pigs inoculated with rBCG–pSO246 or saline. These results show that combined intrarectal and cutaneous inoculation of rBCG–pSOV3J1 in guinea pigs effectively induces high levels of cellular immune responses against the HIV-1 V3J1 antigen.

3.3. Induction of HIV-1 V3J1-specific IgG antibody responses

Sera from guinea pigs inoculated by various routes with rBCG–pSOV3J1 was evaluated by ELISA for antibodies specific for the HIV-1 V3J1 peptide. While IgA responses were not detected in sera from any of the guinea pigs, anti-V3J1 serum IgG responses were induced by nasal and rectal administration of rBCG–pSOV3J1 (Fig. 3A). Anti-V3J1 IgG serum antibodies were measured within 2 weeks in guinea pigs immunized by combined intrarectal and cutaneous inoculations; responses which increased until 4–6 weeks then stabilized (Fig. 3B). These results clearly demonstrate that the combined inoculation of rBCG–pSOV3J1 can be effective for inducing not only cellular but also humoral immune responses specific for HIV-1.

3.4. Optimization of rBCG administration and induction of serum IgA responses

To optimize the administration of rBCG–pSOV3J1, an additional 24 guinea pigs were divided into three groups

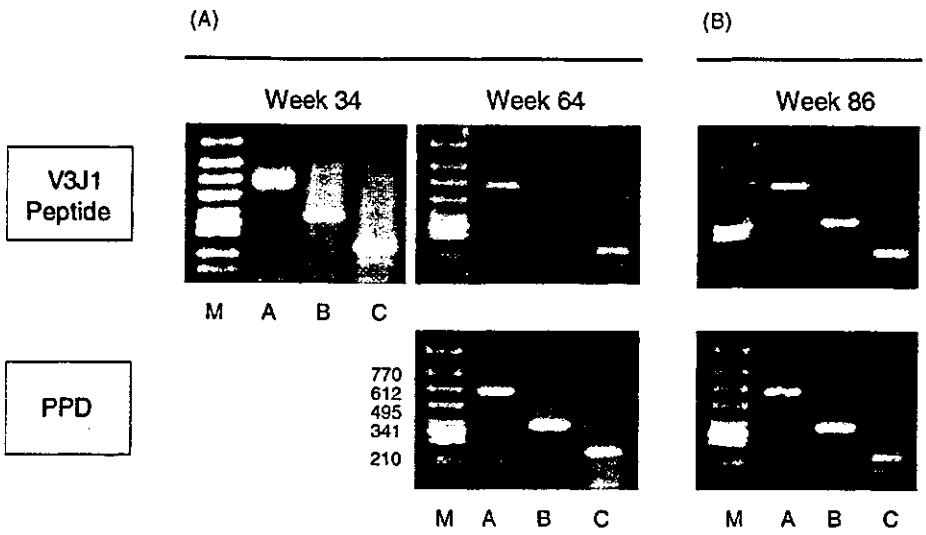

Fig. 5. Expression of cytokine mRNA in PBMC from guinea pigs immunized with rBCG–pSOV3J1. (A) The group received two i.r. (80 mg each) followed by an i.d. (0.1 mg) inoculations. (B) The group received three i.r. (80 mg each) followed by an i.d. (0.1 mg) inoculations. Purified PBMC were stimulated in vitro with V3J1 peptide or PPD for 4 days, and then RNA was extracted. Gene expression was detected by RT-PCR specific for guinea pig cytokines. Lane M, molecular marker; lane A, β-actin; lane B, IFNγ; and lane C, IL-2.

of eight animals. The first group received two i.r. inoculations of rBCG–pSOV3J1 (80 mg each) followed by a s.c. inoculation (1.0 mg). The second group received two i.r. inoculations (80 mg each) followed by an i.d. inoculation (0.1 mg). The remaining eight animals were inoculated with rBCG–pSO246 as a vector control (four animals per group). V3J1-specific IgG responses were detected in the two immunized groups of guinea pigs irrespective of the cutaneous route of administration (s.c. or i.d.) with similar levels of IgG between the two groups (Fig. 4A). Interestingly, anti-V3J1 IgA responses were also detected in the sera of guinea pigs that received combined inoculations of rBCG, although the IgA responses were delayed compared to the induction of IgG (Fig. 4B).

3.5. Enhancement of neutralization activity by a combination of intrarectal and intradermal rBCG inoculation

Serum IgG antibody was obtained from four groups of animals received i.r., i.d., combined i.r./i.d., and a vector control at 3 months after rBCG inoculation. And the mean IC_{50} of serum IgG were 14.6, 68.4, 2.1, and 71.0 μg/ml in i.r., i.d., combined i.r./i.d., and vector control groups, respectively, showing the induction of the neutralization activity in the i.r. groups and the significant enhancement of IC_{50} by the combined regimen.

3.6. Correlation of Th1 cytokines and DTH by intrarectal inoculation of rBCG–pSOV3J1

To further characterize the immune responses induced by combined i.r. and i.d. inoculation of rBCG–pSOV3J1, expression of IFNγ and IL-2 in PBMC from the immunized animals was studied using a cytokine-specific RT-PCR (Fig. 5). In the group of guinea pigs receiving optimized rBCG immunizations, V3J1 peptide-specific IFNγ gene expression was significantly enhanced compared to controls at 34 weeks after initial inoculation, but became undetectable by 64 weeks (Fig. 5A). In contrast, PPD-specific IFNγ gene expression was observed at 64 weeks post-inoculation in the same animals. Furthermore, we found IL-2 gene expression increased in response to V3J1 and PPD antigens at 34 and 64 weeks, respectively. DTH skin reactions specific for V3J1 and PPD were still detected at 66 weeks post-immunization with mean diameters of induration of 10.5 and 11.5 mm, respectively.

V3J1-specific IFNγ and IL-2 mRNA expression was also detected at 86 weeks in a second group of guinea pigs that received three i.r. and an i.d. (0.1 mg) inoculations of rBCG–pSOV3J1 (Fig. 5B). Similarly, mRNA for these two cytokines was also expressed in response to PPD. DTH skin reactions to both V3J1 and PPD antigens were detected in these animals even at 88 weeks with mean diameters of induration of 11.0 and 12.5 mm, respectively. In addition, we investigated antigen-specific IFNγ and IL-2 mRNA expression in mononuclear cells from the peripheral blood, spleen, lungs, and large intestines of these animals at 110 weeks after immunization. As shown in Table 1, IFNγ and IL-2 mRNA was detected in PPD-stimulated PBMC, splenocytes and i-IEL. Moreover, when lymphocytes were stimulated with V3J1-peptide, PBMC, and splenocytes expressed significant levels of IFNγ and IL-2 mRNA, while i-IEL expressed IL-2 mRNA alone. mRNA for these two cytokines was not detected in alveolar lavage cells from the immunized guinea pigs in response to either the V3J1 peptide or PPD.

Table 1
Detection of cytokine mRNA in various tissues from guinea pigs immunized with combined three rectal and an intradermal inoculations of rBCG–pSOV3J1

	PPD		HIV-V3J1	
	IFNγ	IL-2	IFNγ	IL-2
PBMC	+	+	+	+
Spleen	+	+	+	+
Alveolar lavage cell	−	−	−	−
Large intestine	+	+	−	+

Each tissue was collected at 110 weeks after immunization. Purified lymphocytes were stimulated in vitro with V3J1 peptide or PPD for 4 days, and then RNA was extracted to study gene expression by IFNγ- or IL-2-specific RT-PCR. (+) represents cytokine detection and (−) represents no detection.

4. Discussion

We report here on the ability of a vaccine strategy using rBCG–HIV-1 administered by combined rectal and dermal routes to induce both humoral and cellular immune responses against HIV and *M. tuberculosis* in guinea pigs. The vaccine induced DTH reactions to both HIV-V3J1 peptide and tuberculin in immunized guinea pigs, and generated increased titers of HIV-specific serum IgG and IgA antibodies that led to the enhanced neutralization activity against HIV-1_{MN}. In addition, expression of Th1 cytokines in mononuclear cells from peripheral blood, spleen, and large intestine was enhanced for more than 2 years after immunization, suggesting long-term maintenance of antigen-specific memory cells.

Dermal vaccination with BCG is performed widely throughout the world, and has provided protection against tuberculosis, albeit with wide variability in efficacy [32–34], especially in children [35]. Considering the standard route and dose of this vaccine, we attempted to induce enhanced immunity by combining dermal immunization with other routes of inoculation. Using rBCG–pSOV3J1, we combined dermal and intrarectal inoculations to induce immunity against HIV-1 as well as tuberculosis. The rationale behind this strategy was to use dermal immunization with rBCG–pSOV3J1 to provide protection against tuberculosis, while at the same time using intrarectal inoculation to induce antigen-specific mucosal immunity against HIV-1. For these studies, one group of guinea pigs received two i.r. inoculations of rBCG–pSOV3J1 (80 mg each) followed by an i.d. injection (0.1 mg), while a second group received two i.r. inoculations (80 mg each) followed by a s.c. injection (1.0 mg). Our results demonstrate the production of anti-V3J1 serum antibodies and the neutralization activity, at levels significantly higher than those achieved by i.r. inoculation alone. Previous reports showed that serum IgG purified from small animals inoculated with rBCG–HIV neutralized HIV-1, depending on high concentrations of the virus-specific IgG [13–15]. Enhanced antibody responses by the combined regimen therefore are expected to contribute to inactivate HIV-1 more effectively in vivo. Furthermore, significant levels of DTH skin reactions specific for both V3J1 and PPD were detected in guinea pigs that received combined intrarectal/cutaneous inoculations. Thus, the combined regimen appears to efficiently induce systemic cellular and humoral immune responses in the immunized animals. However, the combined i.r./s.c. is designed as a reference regimen against the combined i.r./i.d., because the antigen concentration used for s.c. administration is 10-fold higher than that of authentic BCG vaccination to humans as tuberculosis vaccine. In this study, the combined i.r./s.c. was required for confirming the efficacy associated with different amount of injection with rBCG–HIV and the safety on intrarectal inoculation followed by cutaneous injection. Indeed, no erosions or ulcers were observed at the injection sites when 1.0 mg of rBCG–pSOV3J1 was injected dermally after two rectal inoculations, suggesting the vaccine strategy is safe to administer.

With regard to the induction of mucosal immunity, several reports have shown that secretory IgA is induced in the feces of mice and guinea pigs when rBCG is administered via mucosal routes [18,29,30]. However, in our experiments, rectal IgA was not detected in any of the guinea pigs tested. Our findings are consistent with recent results by Hiroi et al. [15] in which mucosal IgA was not produced in mice by nasal immunization with rBCG–V3J1. Several possible explanations for this discrepancy lie in differences in the dose and time of administration, differences in the BCG strains, and in the species and sex of the animals, any of which might impact on the induction of mucosal immunity. Another possibility is that the induction of IgA may depend on the type of antigens used. For example, the V3 antigen of HIV-1 env is reported to induce antibodies with a low efficiency [19]. In contrast, others have used a rBCG construction that stimulates extremely high humoral and cellular responses against β-galactosidase [18,36]. At the moment, we cannot fully explain the difference in IgA production reported by stimulation with various rBCG immunizations.

In general, the intensity and duration of immune responses induced by immunization are a major consideration for the development of a candidate vaccine. Previous reports have shown an association between HIV clinical progression and the degree of virus-specific DTH responsiveness [37,38]. HIV-specific DTH responders generally had less advanced disease at baseline than non-responders, as judged by CD4 cell count, p24 antibody levels and prevalence of HIV-related symptoms [37,38]. In this study, we observed long-term DTH responses against HIV and tuberculosis antigens at 66 and 88 weeks in guinea pigs immunized with rBCG–pSOV3J1. These results suggest that immunization with rBCG may help maintain HIV-specific DTH responses in vaccinees for an extended period, raising the possibility that this might also confer resistance to disease progression.

Th1 cytokines, in particular IFNγ and IL-2, have been shown to be important for the control of cell-associated pathogens such as HIV and *M. tuberculosis*. Mice with

deletion of the IFNγ gene are much more susceptible to *M. tuberculosis* infection than wild-type mice [39,40], and IFNγ production has been shown to be essential for CD8 cell-mediated protective immunity against *M. tuberculosis* in mice [41]. With respect to HIV infection, treatment of macrophages with IFNγ leads to a reduction in entry and replication of macrophage-tropic strains of HIV [42–44]. To investigate whether mRNA for Th1 cytokines is expressed in guinea pigs immunized with rBCG–pSOV3J1, we developed an RT-PCR assay specific for detection in guinea pigs. Our results show IFNγ and IL-2 mRNA expression at 34 or 86 weeks in antigen-stimulated PBMC from guinea pigs immunized with rBCG–pSOV3J1. In addition, PBMC, splenocytes and i-IEL expressed mRNA for these two cytokines in response to PPD at 110 weeks after vaccination, while PBMC and splenocytes also expressed cytokine-specific mRNA for V3J1 peptide at the same time. IFNγ mRNA expression was not detected in i-IEL stimulated with V3J1 peptide, but was observed in i-IEL stimulated with PPD, indicating that the amount of V3J1 antigen secreted in the intestines may not be sufficient for the induction of Th1-type memory cells specific for V3J1 antigen. It is not clear why antigen-specific IFNγ and IL-2 mRNA were not detected in alveolar lavage cells, but this may reflect an absence of cells expressing these cytokines in the lungs, or alternatively, mRNA expression levels may be below our level of detection.

Can rBCG–HIV be used as a safe vaccine for humans? It has been demonstrated that rBCG–pSOV3J1 has the same levels of stability, toxicity and efficacy against virulent *M. tuberculosis* H37Rv as BCG-Tokyo 172 (Haga and Yamamoto, unpublished results). Since the objective of this study is the development of preventive vaccine, we selected the HIV-1 env V3 region as a model and do not target HIV-infected individuals. However, rBCG–HIV vaccination will be acceptable even if HIV-1-carriers are included in a vaccine-target population with high risk of tuberculosis, because the World Health Organization (WHO) supports the BCG vaccination for asymptomatic HIV-infected children or adults [45]. The combined immunization strategy with rBCG–HIV may greatly facilitate rBCG–HIV administration by inducing potent immune responses against not only tuberculosis but also HIV-1 in humans, which might lead to the possibility of "two vaccines" in "one" recommended by the Expanded Program on Immunization of the WHO [46].

In conclusion, this study provided a more efficient vaccine strategy based on a combination of two routes. High levels of both cellular and humoral immune responses specific for HIV-1 and tuberculosis were induced by combined intrarectal and intradermal vaccination with rBCG–pSOV3J1 at human doses. In addition, antigen-specific IFNγ and IL-2 mRNA expression were detected in the immunized guinea pigs, and PPD-specific Th1-type memory cells were found in the peripheral blood, spleen and large intestine. Cells expressing HIV-specific IFNγ and IL-2 mRNA were maintained for over 2 years after immunization, suggesting that combined i.r. and i.d. vaccination with rBCG–HIV at human doses may confer long-term, antigen-specific immunity in experimental animal models. This kind of approach of immunization with combined use of different routes is practically beneficial to achieve an effective vaccination regimen.

Acknowledgements

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Chimeras of Labile Toxin One and Cholera Toxin Retain Mucosal Adjuvanticity and Direct Th Cell Subsets Via Their B Subunit[1]

Prosper N. Boyaka,[2]* Mari Ohmura,[2]*† Kohtaro Fujihashi,* Toshiya Koga,* Masafumi Yamamoto,†§ Mi-Na Kweon,† Yoshifumi Takeda,¶ Raymond J. Jackson,* Hiroshi Kiyono,*†‡ Yoshikazu Yuki,‡ and Jerry R. McGhee[3]*

Native cholera toxin (nCT) and the heat-labile toxin 1 (nLT) of enterotoxigenic *Escherichia coli* are AB_5-type enterotoxins. Both nCT and nLT are effective adjuvants that promote mucosal and systemic immunity to protein Ags given by either oral or nasal routes. Previous studies have shown that nCT as mucosal adjuvant requires IL-4 and induces CD4-positive ($CD4^+$) Th2-type responses, while nLT up-regulates Th1 cell production of IFN-γ and IL-4-independent Th2-type responses. To address the relative importance of the A or B subunits in $CD4^+$ Th cell subset responses, chimeras of CT-A/LT-B and LT-A/CT-B were constructed. Mice nasally immunized with CT-A/LT-B or LT-A/CT-B and the weak immunogen OVA developed OVA-specific, plasma IgG Abs titers similar to those induced by either nCT or nLT. Both CT-A/LT-B and LT-A/CT-B promoted secretory IgA anti-OVA Ab, which established their retention of mucosal adjuvant activity. The CT-A/LT-B chimera, like nLT, induced OVA-specific mucosal and peripheral $CD4^+$ T cells secreting IFN-γ and IL-4-independent Th2-type responses, with plasma IgG2a anti-OVA Abs. Further, LT-A/CT-B, like nCT, promoted plasma IgG1 more than IgG2a and IgE Abs with OVA-specific $CD4^+$ Th2 cells secreting high levels of IL-4, but not IFN-γ. The LT-A/CT-B chimera and nCT, but not the CT-A/LT-B chimera or nLT, suppressed IL-12R expression and IFN-γ production by activated T cells. Our results show that the B subunits of enterotoxin adjuvants regulate IL-12R expression and subsequent Th cell subset responses. *The Journal of Immunology*, 2003, 170: 454–462.

Native cholera toxin (nCT)[4] and type I heat-labile toxin (nLT) of *Escherichia coli* share 83% amino acid sequence homology and both consist of an enzymatically active A subunit that catalyzes the ADP-ribosylation of the $G_s\alpha i$ protein. The B subunits of CT (CT-B) and LT (LT-B) bind to GM1 gangliosides (1, 2), while LT-B also binds asialo GM1, lactosylceramide, and galactoproteins (3–6). The binding of nCT or nLT to intestinal epithelial cells induces a cascade of events that ultimately results in secretion of chloride and water with subsequent diarrhea (7). Both nCT and nLT are mucosal adjuvants and induce mucosal and systemic immunity when given with protein vaccines by either oral or nasal routes (8–10). Furthermore, A subunit mutants of both CT (11–13) and LT (13–17) have been shown to be devoid of diarrheagenic activity, but to retain full mucosal adjuvanticity, when given by the nasal route.

It has been shown that the adjuvant activity of both nCT and nLT as well as nontoxic mutants of CT involves up-regulation of costimulatory molecule expression by APCs (18–21). On the other hand, CT-B and LT-B fail to stimulate APC costimulatory molecule expression (19, 21). In addition, studies have now shown that neither CT-B nor LT-B enhances immune responses to mucosally coadministered protein Ags (2, 14, 21, 22). However, some reports have suggested that CT-B and LT-B display mucosal adjuvant activity when large doses are given with proteins by the nasal route (2, 13, 23, 24) or when enterotoxin B subunits are directly conjugated to the Ag itself (2, 23). Previous studies have shown that nCT as adjuvant elicits potent mucosal and systemic $CD4^+$ Th2-type immune responses (25–28). Proof that IL-4 is involved in nCT-induced mucosal secretory IgA (S-IgA) Ab responses to coadministered protein Ags was provided by the finding that nCT fails to induce mucosal S-IgA Ab responses in IL-4 gene knockout ($IL\text{-}4^{-/-}$) mice (25, 27, 28). This ability of nCT to promote polarized Th2-type responses is in part explained by recent findings that nCT inhibits IL-12 production by monocytes and dendritic cells (19, 29) and abrogates IL-12R expression by T cells (29). It has also been shown that nLT as mucosal adjuvant supports $CD4^+$ Th1-type responses and IFN-γ production in the presence of IL-4-independent Th2-type responses (20, 30). It is clear that an in vivo network of Th1- or Th2-type cytokine responses can influence the overall nature of the immune response seen (31, 32). Thus, Th1-type cytokines support cell-mediated immunity and the production of complement-fixing IgG subclass Abs. On the other hand, Th2-type cytokines provide help for B cells and promote the production of IgE and noncomplement fixing, IgG1 and IgG2b

*Departments of Microbiology and Oral Biology, Immunobiology Vaccine Center, University of Alabama, Birmingham, AL 35294; †Department of Mucosal Immunology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; ‡Division of Mucosal Immunology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan; §Department of Oral Medicine, Nihon University School of Dentistry at Matsudo, Chiba, Japan; and ¶Department of Microbiology, Jissen Women's College, Tokyo, Japan

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[2] P.N.B. and M.O. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Jerry R. McGhee, Department of Microbiology and Immunobiology Vaccine Center, University of Alabama, BBRB 761, 845 19th Street South, Birmingham, AL 35294-2170. E-mail: mcghee@uab.edu

[4] Abbreviations used in this paper: nCT, native cholera toxin; AFCs, Ab-forming cells; CLN, cervical lymph nodes; CT, cholera toxin; CT-A, CT A subunit; CT-B, CT B subunit; LT, *E. coli* heat-labile toxin I; LT-A, LT A subunit; LT-B, LT B subunit; S-IgA, secretory IgA.

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subclass Abs. Furthermore, CTL responses were induced after nasal immunization with either nCT or nLT as adjuvant (33, 34); however, higher CTL responses were noted after immunization with nLT or its nontoxic derivatives compared with nCT (35, 36). This suggests that a potent Th1 cell component characterizes the immune response induced when nLT is used as mucosal adjuvant.

Two recent studies suggest that slight alterations in the A subunits explain the differences in nCT and nLT adjuvanticity (37, 38). However, a major difference between nCT and nLT involves their B subunit ganglioside receptors, with nLT being more promiscuous. We reasoned that this could also explain the differences in Th1- or Th2-type responses induced by nLT or nCT, respectively. To address the relative contributions of the A and B subunits in Th1- vs Th2-type responses, we constructed the appropriate chimeras, i.e., CT-A/LT-B and LT-A/CT-B. These chimeras were assessed for mucosal adjuvant activity after nasal codelivery with protein Ags, and the results show that both A and B subunits contribute to adjuvanticity, with the B subunit associated with Th cell subset demarcation.

Materials and Methods

Construction of CT-A/LT-B and LT-A/CT-B chimeras

Enterotoxin chimeras were generated by spontaneous association of the A subunit of nCT or nLT with the B subunit of the opposite enterotoxin. The CT-A subunit was purchased from Sigma-Aldrich (St. Louis, MO) and applied to an immobilized D-galactose column (Pierce, Rockford, IL) to remove any CT-B contamination. Eluted CT-A contained no B subunit, as shown by silver staining of SDS-PAGE gels. Recombinant CT-B was produced using a *Brevibacillus choshinensis*-expressing plasmid pNU 212-CTB (39) and was provided by JCR Biopharmaceuticals (San Diego, CA). LT-B was derived from an *E. coli* K12 strain DH5α transformant containing the plasmid (pLT10) that encodes the LT-B gene (40) and was purified by use of an immobilized D-galactose gel column. The nCT was obtained from List Biological Laboratories (Campbell, CA), and nLT was purchased from Sigma-Aldrich. The CT-A/LT-B and LT-A/CT-B chimeras were generated by spontaneous association of A and B subunits in propionic acid, as previously described (41). Briefly, CT-A and LT-B were separately dialyzed in 0.1 M propionic acid (pH 4.0). A 2-fold molar excess of CT-B was then mixed with LT-A, and the two were allowed to spontaneously associate during a 24-h dialysis in 0.1 M propionic acid (pH 4.0). The associated CT-A/LT-B chimera was purified by gel filtration. The LT-A/CT-B chimera was generated by the same procedure used to construct the opposite chimera. Briefly, nLT was first denatured by dialysis in 0.1 M propionic acid (pH 4.0), followed by a second dialysis step in 0.1 M propionic acid/6 M urea, to separate the A and B subunits. The A subunit was then purified by HPLC using a TSK gel 2000 W (Tosoh Biosep, Montgomeryville, PA) and 0.1 M propionic acid/6 M urea plus 0.2 M NaCl as eluent. The urea was removed by dialysis in 0.1 M propionic acid before LT-A and CT-B were mixed at a 1:2 molar ratio and allowed to spontaneously associate. The resulting LT-A/CT-B chimera was purified by gel filtration as previously reported (42).

Mice and nasal immunization

C57BL/6 mice were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD). Mice were maintained in horizontal laminar flow cabinets and were free of microbial pathogens as determined by plasma Ab screening and tissue histopathology performed on sentinel mice. All mice received sterile food and water ad libitum and were between 8 and 12 wk of age when used for these experiments.

Mice were nasally immunized at weekly intervals for 3 consecutive wk with 100 μg of OVA (Sigma-Aldrich) either alone or together with 0.5 μg of nCT, nLT, or CT-A/LT-B or LT-A/CT-B chimeras (12, 43). The nasal immunization was performed on lightly anesthetized mice with OVA and adjuvant in a total volume of 10 μl, with 5 μl placed into each nare. Blood samples were collected weekly (days 7, 14, and 21) just before reimmunization to monitor the development of plasma anti-OVA Ab responses as previously described (12, 43). Mucosal secretions (e.g., fecal extracts, nasal washes, and saliva) were collected on day 21 as previously described (12, 43) for assessment of mucosal S-IgA Ab responses.

Evaluation of OVA-specific Ab isotypes and IgG subclass responses

OVA-specific Ab titers in plasma and mucosal secretions were determined by ELISA as previously described (12, 25, 44). Briefly, serial 2-fold dilutions of plasma or mucosal secretions were added to plates coated with OVA (1 mg/ml). Anti-OVA Ab isotypes were detected with peroxidase-labeled goat anti-mouse μ, γ, or α H chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL). For IgG subclass analysis, biotinylated rat anti-mouse γ1 (clone G1-7.3), γ2a (clone R19-15), γ2b (clone R12-3), or γ3 (clone R40-82) H chain-specific mAbs (BD PharMingen, San Diego, CA) and streptavidin-conjugated peroxidase were employed. The colorimetric reaction was developed by the addition of ABTS substrate (Sigma-Aldrich). End-point titers were expressed as the reciprocal $\log_2$ dilution giving an OD_{415} of $\leq$0.1 above those obtained with control, nonimmunized mice.

Total plasma IgE levels were determined by ELISA as previously described (25, 26, 45) using a rat anti-mouse IgE mAb (BD PharMingen; clone R35-72) and a second biotinylated rat anti-mouse IgE mAb (BD PharMingen; clone R35-92) for capture and detection, respectively. The OVA-specific plasma IgE Ab responses were detected using a streptavidin-poly-HRP amplification system (Research Diagnostics, Flanders, NJ) and the ABTS substrate (44). A modified IgE capture luminometry assay (12, 46) was also used, and the end-point titers were determined as the sample dilution exhibiting relative light units which were 2-fold higher than background.

ELISPOT assay for detection of Ab-forming cells (AFC)

OVA-specific AFC in mucosal and systemic tissues were evaluated as previously described (12, 45, 47). Dispersed cells were resuspended in RPMI 1640 medium (Cellgro; Mediatech, Washington, DC) containing 10% FCS, 15 mM HEPES, 2 mM L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin (complete medium). Different dilutions of cells were then added to 96-well nitrocellulose-based plates (Millipore, Bedford, MA) coated with 100 μl of a 1 mg/ml solution of OVA and incubated for 6 h at 37°C in a 5% CO_2 atmosphere. Ag-specific AFC were detected with peroxidase-labeled anti-mouse μ-, γ-, or α-chain Abs (Southern Biotechnology Associates). Spots were visualized by adding the chromogenic substrate, 3-amino-9-ethylcarbazole (Moss, Pasadena, MD) and were counted with the aid of a dissecting microscope (SZH Zoom Stereo Microscope System; Olympus, Lake Success, NY).

Ag-specific CD4$^+$ T cell and cytokine responses

Single-cell suspensions from the spleen and from cervical lymph nodes (CLN) were obtained as previously described (12, 44). The cells were first added to a nylon wool column (Polysciences, Warrington, PA) and incubated for 1 h at 37°C to obtain an enriched T cell fraction. The nonadherent, T cell-enriched population was stained with biotinylated anti-CD4 mAb (clone GK1.5), followed by streptavidin-coupled microbeads (MACS; Miltenyi Biotec, Auburn, CA). The CD4$^+$ T cells were then obtained at >98% purity by positive sorting using a MACS system (Miltenyi Biotec). In some experiments CD4$^+$ T cells were enriched (>98% purity) using Mouse CD4 Collect Plus columns (Biotex, Edmonton, Canada). Purified CLN and splenic CD4$^+$ T cells were cultured at a density of 4×10^6 cells/ml and stimulated with OVA (1 mg/ml) in the presence of T cell-depleted, irradiated (3000 rad) splenic feeder cells (8×10^6 cells/ml) and IL-2 (10 U/ml; BD PharMingen) in complete medium. To measure cell proliferation, 0.5 μCi of tritiated thymidine ([^{3}H]TdR; DuPont/NEN, Boston, MA) was added to individual culture wells 4 days later. Eighteen hours after the addition of [^{3}H]TdR, the cells were harvested onto glass microfiber filter paper (Whatman, Clifton, NJ), and [^{3}H]TdR incorporation was determined by liquid scintillation counting.

Analysis of OVA-specific CD4$^+$ T cell cytokine responses

Supernatants from OVA-stimulated CD4$^+$ T cell cultures were collected after 5 days of incubation, and cytokine levels were determined by ELISA as described previously (12, 26, 44, 45, 48). Briefly, Nunc MaxiSorp Immunoplates (Nunc, Naperville, IL) were coated with anti-mouse IFN-γ (clone R4-6A2), IL-2 (clone JES6-1A12), IL-4 (clone BVD4-1D11), IL-5 (clone TRFK5), IL-6 (clone MP5-20F3), or IL-10 (clone JES5-2A5) mAbs (BD PharMingen) in 0.1 M sodium bicarbonate buffer (pH 8.2). After blocking, cytokine standards and serial dilutions of culture supernatants were added in duplicate. The plates were washed and incubated with secondary biotinylated anti-mouse IFN-γ (clone XMG-1.2), IL-2 (clone JES6-5H4), IL-4 (clone BVD6-24G2), IL-5 (clone TRFK4), IL-6 (clone MP5-32C11), or IL-10 (clone JES5-16E3) mAbs (BD PharMingen), followed by peroxidase-labeled goat anti-biotin Ab (Vector Laboratories, Burlingame,

CA). The color was developed with ABTS as described above. Standard curves were generated using mouse rIFN-γ, rIL-5, rIL-6, and rIL-10 (R&D Systems, Minneapolis, MN), rIL-2 (BD PharMingen), and rIL-4 (Endogen, Boston, MA). The ELISAs were capable of detecting 5 pg/ml of IL-2, IL-4, and IL-5, 15 pg/ml IFN-γ and 20 pg/ml IL-6 and IL-10.

Flow cytometric analysis of IL-12Rβ1 expression

Splenic T cells were isolated from naive mice and seeded into culture plates at a density of 5×10^6 cells/ml. To investigate the effects of nCT, nLT, and the two chimeras on IL-12Rβ1 expression, T cells were incubated for 1 h with 100 ng/ml of nCT, nLT, LT-A/CT-B, or CT-A/LT-B. Anti-mouse CD3ε chain (1 μg/ml; clone 145-2C11; BD PharMingen) was then added. After 3 days of incubation the T cells were incubated for 30 min at 4°C with PE-labeled anti-mouse IL-12Rβ1 (clone 114) and FITC-labeled anti-mouse CD3 (clone 145-2C11) or anti-CD4 (clone GK1.5). Cells were then washed and fixed in 1% paraformaldehyde in PBS and analyzed by flow cytometry using a FACSCalibur equipped with the CellQuest software (BD Biosciences, Mountain View, CA).

Statistics

The results shown are reported as the mean ± 1 SE. Statistical significance ($p < 0.05$) was determined by Student's t test and the Mann-Whitney U test for unpaired samples. The results were analyzed using the StatView II statistical program (Abacus Concepts, Berkeley, CA) for Apple computers (Cupertino, CA).

Results

Adjuvant activity of nasal CT-A/LT-B and LT-A/CT-B chimeras for serum Ab responses

We initially determined whether chimeric molecules generated by spontaneous association of the A subunit of CT or LT and the B subunit of the reciprocal enterotoxin would display adjuvanticity when nasally coadministered with a weak protein Ag, i.e., OVA. For this purpose, mice were nasally immunized with OVA and CT-A/LT-B, LT-A/CT-B, nCT, or nLT as mucosal adjuvants. Both CT-A/LT-B and LT-A/CT-B enhanced plasma anti-OVA Ab isotype responses, demonstrating that both chimeras were effective adjuvants for nasally coadministered OVA (Fig. 1*A*). No significant differences were noted in the levels of plasma IgM, IgG, or IgA anti-OVA Ab responses induced by the two chimeras compared with the two native enterotoxins (Fig. 1*A*). Since nCT and nLT promote distinct patterns of IgG Ab subclass responses to mucosally coadministered protein Ags (20, 25, 26, 28, 30, 45), we next investigated the patterns of plasma anti-OVA IgG subclass Ab responses resulting from the adjuvant activity of the two chimeras compared with the native enterotoxins. The plasma IgG Ab subclass responses in mice that received OVA and nLT or the CT-A/LT-B chimera were characterized by high anti-OVA IgG2a, with IgG1 and IgG2b Ab responses (Fig. 1*B*). On the other hand, mice that received the nasal vaccine consisting of OVA and nCT or LT-A/CT-B showed high levels of IgG1, followed by IgG2b, and only low levels of IgG2a anti-OVA Abs (Fig. 1*B*). These results clearly show that both CT-A/LT-B and LT-A/CT-B chimeras are mucosal adjuvants, and their activities mimic the native enterotoxin with which they share a common B subunit.

To further elucidate differences between CT-A/LT-B and LT-A/CT-B chimeras, we next analyzed their potential to promote plasma IgE Ab responses. Previous studies in mice orally or nasally immunized three times at weekly intervals with protein vaccines and nCT as mucosal adjuvant have shown that a transient IgE Ab response occurs by 7 days following the second immunization (day 14) (12, 20, 25, 26, 45). It has also been shown that nLT induces lower IgE Ab responses compared with nCT (20, 30). In this study we noted that both CT-A/LT-B and LT-A/CT-B chimeras induced plasma IgE Ab responses (Table I). These IgE responses were higher in mice given either LT-A/CT-B or nCT compared with mice that received CT-A/LT-B or nLT as nasal adjuvants (Table I). Interestingly, no significant differences were noted between Ag-specific IgE Ab responses induced by nCT or LT-A/CT-B. On the other hand, the anti-OVA IgE Ab responses elicited by CT-A/LT-B were slightly higher than those induced by nLT; however, the differences were not statistically significant (Table I).

Both CT-A/LT-B and LT-A/CT-B chimeras support mucosal S-IgA Ab responses

Mucosal secretions from mice nasally immunized with OVA in the presence of CT-A/LT-B or LT-A/CT-B were analyzed to determine whether these chimeric enterotoxins also induced mucosal S-IgA Ab responses. No significant anti-OVA S-IgA Ab responses were seen in the saliva of mice given OVA alone. In contrast, higher levels of salivary IgA anti-OVA Abs were noted in mice that received OVA with either CT-A/LT-B or LT-A/CT-B as mucosal adjuvant (Fig. 2*A*). Similar levels of IgA anti-OVA Abs were

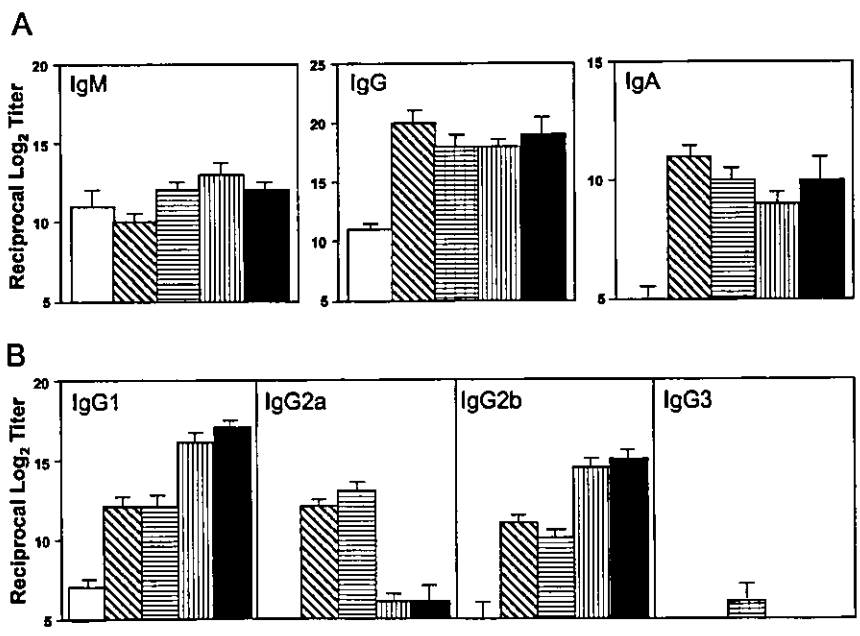

FIGURE 1. Plasma anti-OVA Ab isotypes and IgG subclass responses following nasal immunization with CT-A/LT-B or LT-A/CT-B chimeras. *A*, The levels of plasma anti-OVA IgM, IgG, and IgA Ab responses are shown; *B*, the levels of anti-OVA IgG subclass Ab responses were evaluated by ELISA 1 wk after the last immunization (day 21). Mice were immunized three times at weekly intervals with OVA only (□), OVA plus CT-A/LT-B (▨), OVA plus nLT (▤), OVA plus LT-A/CT-B (▥), or OVA plus nCT (■). The results are expressed as the reciprocal $\log_2$ titers ± 1 SE from four separate experiments and five mice per group per experiment.

Table I. *Total and OVA-specific IgE Ab responses in mice nasally immunized with OVA and chimeric or native enterotoxins*[a]

Nasal OVA Plus Adjuvant	Total IgE (μg/ml)	OVA-Specific IgE (reciprocal $\log_2$ titers)
None	Below detection[b]	Below detection[b]
CT-A/LT-B	2.3 ± 0.4	7.5 ± 1.2
nLT	1.9 ± 0.1	6.0 ± 0.6
LT-A/CT-B	6.5 ± 0.3	11.1 ± 0.7
nCT	8.6 ± 0.6	13.6 ± 0.4

[a] Groups of five mice were nasally immunized with 100 μg of OVA and 0.5 μg of chimera or native enterotoxin three times at weekly intervals (days 0, 7, and 14). Total and OVA-specific plasma IgE levels were determined on day 14 as described in *Materials and Methods*. Results are from one experiment and are representative of three separate experiments.

[b] Below the limits of detection. Total IgE, <150 ng/ml; OVA-specific IgE, <4 reciprocal $\log_2$ titer.

induced in this secretion by both nCT and nLT (Fig. 2*A*). We also compared mucosal S-IgA Ab responses induced by chimeras and nCT or nLT in nasal washes. Both CT-A/LT-B and LT-A/CT-B induced similar levels of S-IgA anti-OVA Abs, which were not significantly different from those induced by nCT or nLT (Fig. 2*B*).

To establish the mucosal origin of anti-OVA IgA Abs seen in these external secretions, we next evaluated numbers of IgA AFCs in the CLN of nasally immunized mice. High numbers of OVA-specific IgA AFCs were detected in CLNs of mice nasally immunized with OVA and nCT, nLT, or chimeras as mucosal adjuvants (Fig. 3*A*). Analysis of splenic OVA-specific AFCs of mice nasally immunized with OVA and either CT-A/LT-B or LT-A/CT-B as mucosal adjuvants showed higher numbers of OVA-specific IgG compared with IgA AFCs, which is consistent with higher levels of serum OVA-specific IgG than IgA Abs (Fig. 3*B*). The frequency of IgA AFCs in mucosal tissues of mice nasally immunized with the chimeras was of the same magnitude as that in mice induced by nCT and nLT. Further, higher numbers of IgA AFCs were noted in the CLNs compared with spleen (Fig. 3).

Distinct CD4$^+$ Th cell subsets are induced by CT-A/LT-B and LT-A/CT-B chimeras

The pattern of IgG subclass responses elicited by the two chimeras suggests that separate mechanisms account for adjuvanticity. Further, the results show that LT-like immunity was induced by CT-A/LT-B, while LT-A/CT-B supported CT-like immune responses. To clarify the precise Th cell subset pathways associated with the Ab responses elicited by CT-A/LT-B or LT-A/CT-B as adjuvant, we next analyzed the profile of CD4$^+$ Th1- and Th2-cell cytokine responses supported by these two chimeras. We noted that OVA-specific CD4$^+$ T cells from CLNs and spleen of mice nasally immunized with OVA and either nLT or CT-A/LT-B secreted high levels of IFN-γ after in vitro restimulation with OVA (Fig. 4). On the other hand, only minimal levels of IFN-γ were seen in culture supernatants of OVA-stimulated CLN and splenic CD4$^+$ T cells isolated from mice immunized with OVA and LT-A/CT-B or nCT as mucosal adjuvants (Fig. 4).

Previous studies have shown that nCT induces significant IL-4 responses compared with nLT (20, 30). Further, the mucosal adjuvant activity of nCT, but not nLT, requires IL-4, and no adjuvant effect was seen in IL-4 knockout mice (25, 27, 28). Therefore, we compared the ability of our chimeric enterotoxins to support IL-4 and CD4$^+$ Th2-type responses. Analysis of culture supernatants of OVA-stimulated CD4$^+$ T cells from CLNs and spleen of mice nasally immunized with OVA and native enterotoxins or with the chimeras showed no significant differences in levels of IL-5, IL-6,

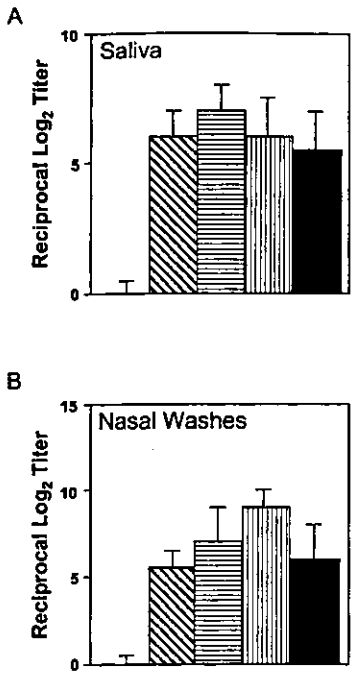

FIGURE 2. Anti-OVA S-IgA Ab responses in mucosal secretions following nasal immunization with CT-A/LT-B or LT-A/CT-B chimeras. Saliva (*A*) and nasal washes (*B*) were collected on day 21 from mice nasally immunized with OVA only (□), OVA with CT-A/LT-B (▨), nLT (▤), LT-A/CT-B (▥), or nCT (■). OVA-specific IgA Ab responses were evaluated by ELISA. The results are expressed as individual end-point ($\log_2$) Ab titers ± 1 SE from four experiments each with five mice per group per experiment.

and IL-10 (Fig. 5). Conversely, the adjuvant activity of CT-A/LT-B and LT-A/CT-B differentially affected IL-4 synthesis by OVA-specific CD4$^+$ T cells. Thus, the cultures of CD4$^+$ T cells from mice nasally immunized with OVA and LT-A/CT-B as adjuvant produced high levels of IL-4, which were essentially identical with those seen in the cultures of CD4$^+$ T cells from mice immunized with OVA and nCT (Fig. 5). On the other hand, significantly lower and similar levels of IL-4 production were seen in culture supernatants of CD4$^+$ T cells from mice that received CT-A/LT-B or nLT as nasal adjuvants (Fig. 5). Taken together, these results show that the induction of CD4$^+$ Th2-type responses by LT-A/CT-B closely mimics that induced by nCT, while Th1- and select Th2-type responses were promoted by both CT-A/LT-B and nLT.

Both LT-A/CT-B and nCT inhibit T cell IL-12Rβ1 expression

We next addressed the potential mechanism involved in the induction of biased Th cell cytokine responses by nCT, nLT, and the chimeras LT-A/CT-B and CT-A/LT-B by analyzing the effects of these enterotoxins on IL-12Rβ1 expression and IFN-γ secretion by activated T cells. Addition of anti-CD3 mAb to splenic T cell cultures enhanced IL-12Rβ1 expression (Fig. 6). Pretreatment of splenic T cells with nCT, but not nLT, abolished the anti-CD3 mAb-induced IL-12Rβ1 expression (Fig. 6). When cells were pretreated with the LT-A/CT-B chimera, we observed an inhibition of IL-12Rβ1 expression similar to that induced when cells were pretreated with nCT (Fig. 6). Conversely, no inhibition of IL-12Rβ1 expression was seen in spleen cell cultures pretreated with the CT-A/LT-B chimera (Fig. 6), again suggesting that chimeric enterotoxins reproduce the effect of the native enterotoxin with which they share the same B subunit. Neither CT-B nor LT-B alone affected IL-12Rβ1 expression by anti-CD3 mAb-stimulated T cells

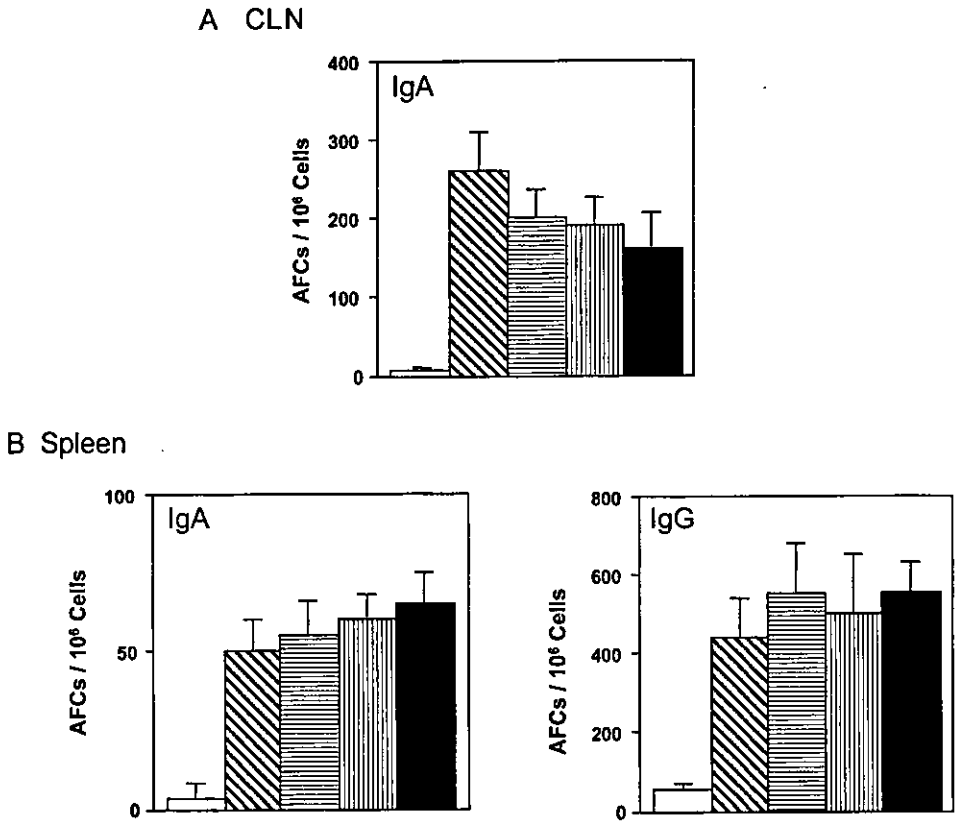

FIGURE 3. Anti-OVA AFCs in CLN (*A*) and spleen (*B*) following nasal immunization with CT-A/LT-B or LT-A/CT-B chimera. The CLN and spleen cells were isolated 21 days after the initial immunization with OVA only (□), OVA with CT-A/LT-B (▨), nLT (▤), LT-A/CT-B (▥), or nCT (■) and subjected to OVA-specific ELISPOT assay for enumeration of IgA or IgG AFCs. The results are expressed as mean AFCs ± 1 SE and are representative of four separate experiments, each with five mice per group per experiment.

(not shown). To further establish the functional relevance of the adjuvant effects of nCT, nLT, and the chimeric enterotoxins on IL-12Rβ1 expression, we investigated how pretreatment with enterotoxin would affect IFN-γ secretion by anti-CD3 mAb-stimulated T cells. The ability of anti-CD3 mAb-stimulated T cells to produce IFN-γ was essentially abolished by pretreatment of T cells with nCT (Fig. 7). In contrast, pretreatment with nLT only marginally affected IFN-γ production by anti-CD3 mAb-stimulated T cells (Fig. 7). Interestingly, pretreatment of cells with LT-A/CT-B altered IFN-γ production, while CT-A/LT-B had no significant effect on this response (Fig. 7).

Discussion

Both nCT and nLT have been extensively used as mucosal adjuvants in various animal models; however, little is known about the precise mechanisms that govern the nature of $CD4^+$ Th cell responses induced by these related molecules for support of mucosal and plasma Ab responses. We have investigated the involvement of the A and B subunits of these two enterotoxins in $CD4^+$ Th cell subset responses by analyzing the patterns of immune responses promoted by the reciprocal CT-A/LT-B or LT-A/CT-B chimeras compared with the parent nCT or nLT. Both chimeras were effective adjuvants for a nasally coadministered protein Ag, OVA, which is poorly immunogenic when given alone. Both chimeras elicited mucosal and plasma Ab responses similar in magnitude to those induced when nCT or nLT was used as mucosal adjuvant. Further, the adjuvant activity of CT-A/LT-B resulted in OVA-specific IgG subclass and $CD4^+$ Th cell responses identical with those induced by nLT. In contrast, identical IgG subclass and $CD4^+$ Th2-type responses were induced by LT-A/CT-B and nCT, clearly indicating that the B subunit portion of CT and LT dictates the nature of Th cell subset responses induced by these mucosal adjuvants. A role for the B subunit in the development of Th1- or Th2-type responses was further supported by the finding that LT-A/CT-B, like nCT, inhibited IL-12Rβ1 expression and IFN-γ production by T cells, while these effects were not seen after treatment with nLT or the chimera CT-A/LT-B.

The first important finding of this study was that both the A and B subunits of CT and LT could be freely substituted without altering their ability to serve as mucosal adjuvants. Chimeras generated by genetic expression of the A subunit of one enterotoxin and the B subunit of the other were reported to stimulate plasma IgG Ab responses at levels comparable to those achieved by native enterotoxins (37). In addition, our recent and separate study showed that a nontoxic CT-A-E112K/LT-B chimera produced with a *Brevibacillus choshinensis* expression system was an effective adjuvant for mucosal and systemic immunity (42). Here we provide evidence that CT-A/LT-B and LT-A/CT-B chimeras, which were generated by spontaneous reassociation of A and B subunits, also retain their mucosal adjuvant activity. Despite the close similarity between nCT and nLT (1), there are subtle functional differences between these two molecules. The stability and conformation of these enterotoxins are important factors in their adjuvant activity, and thus one could not exclude the possibility that altered conformation or stability would affect the adjuvanticity of the chimeras. In this regard, CT-A2 was reported to more efficiently stabilize interactions between the A and B subunits than did LT-A2 (49). Further, the same mutation in CT or LT differentially affected their adjuvant activity. For example, a nontoxic LT mutant with a serine to lysine substitution at position 63 in the A subunit (LTK63) was shown to be an excellent mucosal adjuvant (13, 16, 50, 51); however, the CT mutant bearing the same single amino acid substitution (CTK63) did not display adjuvant activity (14). It has also been shown that receptor binding mutants of LT (LT-B G33D) fail to evoke systemic and mucosal immune responses (52).

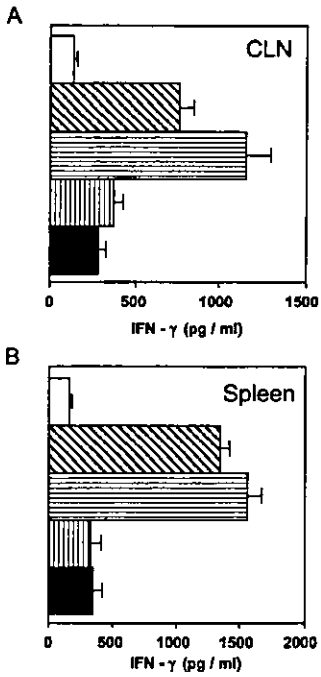

FIGURE 4. The production of IFN-γ by OVA-specific $CD4^+$ T cells from mice nasally immunized with chimeric CT-A/LT-B or LT-A/CT-B enterotoxins. The CLN (*A*) and splenic (*B*) $CD4^+$ T cells were isolated 21 days after the initial immunization with OVA only (□) or OVA with CT-A/LT-B (▨), nLT (▤). LT-A/CT-B (▥), or nCT (■). After in vitro restimulation with OVA, culture supernatants were subjected to IFN-γ-specific ELISA. The results are expressed as the mean ± 1 SE and are representative of five separate experiments, each with five mice per group per experiment.

Further, higher doses of nontoxic mutants of LT or CT were required to induce similar response levels as native enterotoxins (2, 12, 13, 16, 43, 51). Our findings that both CT-A/LT-B and LT-A/CT-B possess adjuvant activity indicate that the A and B subunits of CT and LT can be interchanged, and the resulting chimeric enterotoxins retain both ganglioside binding and ADP ribosyltransferase activities. In contrast to studies suggesting an essential role of A subunits in Th cell subset responses, our findings support a major role for the B subunits in directing the nature of Th cell cytokine responses to coadministered protein Ags. This possibility was initially suggested by separate studies in which the nontoxic CT-A-E112K/LT-B chimera exhibited an LT-like adjuvant activity (42). However, the single amino acid substitution in the A subunit of this chimera could also have influenced the profile of the Th cell cytokine responses induced.

Earlier studies comparing the receptors for nCT and nLT indicated that while both molecules bound GM1 and, to a lesser extent, GD1b, nLT bound other gangliosides as well as glycoproteins (6, 53, 54). Studies performed in rabbit small intestine revealed that blocking of GM1 and GD1b with CT-B did not affect the binding of nLT to glycoproteins and subsequent intestinal fluid secretion (4, 53). In the human small intestine, extraction of lipids from intestinal epithelial cells resulted in removal of all nCT binding sites, but only 50% of the nLT binding sites (54). Moreover, nLT was found to bind polyglycosylceramides in rabbits, while nCT did not (5). Thus, this pleiotrophic binding to the above sites probably contributes to the Th1 component of the LT-induced immune response.

It is now well documented that nCT as a mucosal adjuvant promotes $CD4^+$ Th2-type responses to coadministered protein Ags (12, 21, 25–28, 45, 55). The key role played by IL-4 was demonstrated by the loss of nCT-induced mucosal adjuvanticity in IL-4 knockout mice (27, 28). In vitro studies have also shown that nCT inhibits IL-12 production by human monocytes and DCs (29). Interestingly, these studies showed that higher doses of nLT were required to inhibit IL-12 production (29). These observations add support to in vivo studies that showed predominant Th2-type responses after mucosal immunization with nCT (12, 20, 25–28, 45, 55) and Th1- with low IL-4 Th2-type responses when nLT was used as the mucosal adjuvant (17, 20, 30). However, the precise role of the A vs B subunits in adjuvanticity had not been studied in detail. In this regard, CT-B, like nCT, was reported to stimulate the synthesis of arachidonic acid metabolites (56) including PGE_2, which is known to promote the differentiation of Th2-type cells

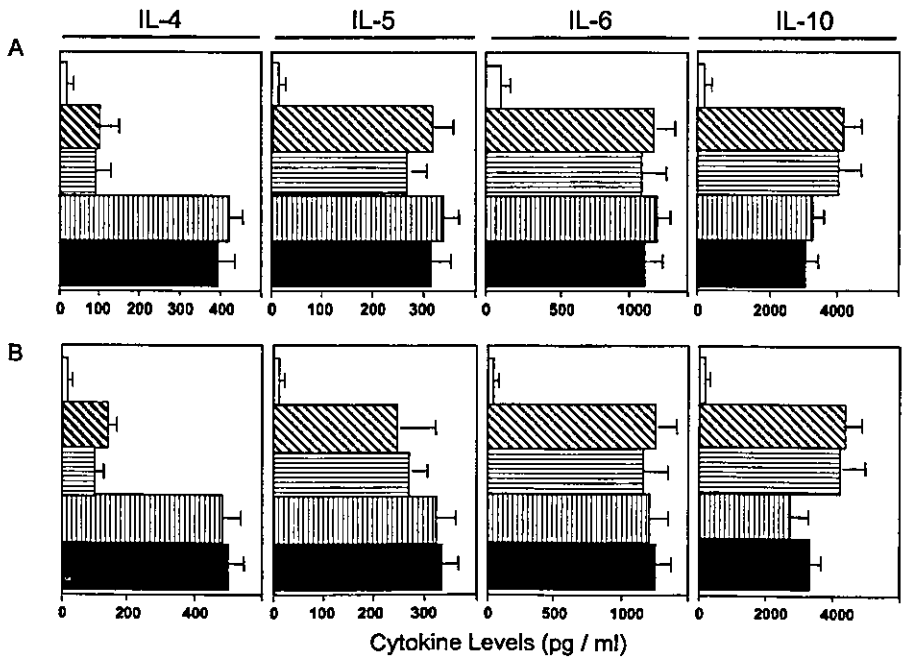

FIGURE 5. The production of Th2-type cytokines by OVA-specific $CD4^+$ T cells from mice nasally immunized with chimeric CT-A/LT-B and LT-A/CT-B enterotoxins. The CLN (*A*) and splenic (*B*) $CD4^+$ T cells were isolated 21 days after the initial immunization with OVA only (□), OVA with CT-A/LT-B (▨), nLT (▤), LT-A/CT-B (▥), or nCT (■). After in vitro restimulation, Th2-type cytokine secretion in culture supernatants was evaluated by IL-4-, IL-5-, IL-6-, and IL-10-specific ELISA. The results are expressed as the mean ± 1 SE and are representative of five separate experiments, each with five mice per group per experiment.

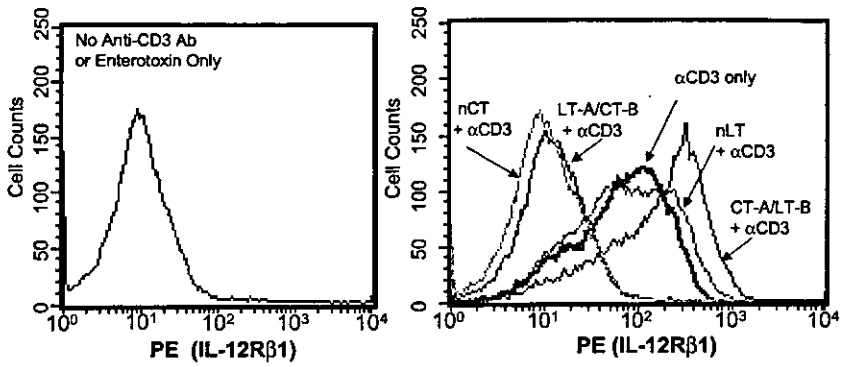

FIGURE 6. Treatment with nCT and LT-A/CT-B inhibits IL-12Rβ1 expression by anti-CD3 mAb-stimulated T cells. Naive splenic T cells were treated for 1 h with nCT, nLT, CT-A/LT-B, or LT-A/CT-B (100 ng/ml) and then were cultured for 3 days in the presence of anti-CD3 mAb (1 μg/ml). Control T cells were either not treated with enterotoxin nor stimulated with anti-CD3 mAb (*left panel*) or were only stimulated with anti-CD3 mAb (*right panel*). Flow cytometry was performed after staining with PE-labeled anti-mouse IL-12Rβ1 and FITC-labeled anti-mouse CD3 or anti-CD4 mAbs. Data are representative of four separate experiments.

(57–60). While this would indicate a predominant role of the binding B subunit for the induction Th2-type responses, other reports favor a role for the A subunit. For example, single amino acid substitutions in the A subunit of LT (LTK63 and LTR72 mutants) were shown to promote protein Ag-specific mixed Th1/Th2- or Th2-type responses, respectively (38). Another study showed that genetically produced CT-A/LT-B and LT-A/CT-B as nasal adjuvants induced IFN-γ and IL-5 secretion in vitro, and these authors suggested that the A subunits (i.e., CT-A and LT-A) of these adjuvants actually controlled the nature of $CD4^+$ Th cell cytokine responses induced (37). Unfortunately, this study did not investigate the complete pattern of Th2-type cytokines, including IL-4, which is the major cytokine for Th2 cells differentially affected by nCT compared with nLT. It is important to note that immune responses induced by native (i.e., nCT and nLT) or chimeric (CT-A/LT-B or LT-A/CT-B) enterotoxins could be different if these enterotoxins were used as adjuvants for more complex Ags or Ags with intrinsic biological activity (i.e., endotoxin). In this regard, high IgG1 and IgG2b Ab responses to *Hemophilus influenzae* were seen in mice immunized nasally with a recombinant outer membrane protein P6 of nontypeable *H. influenzae* and nCT as adjuvant (61). In contrast, no IgG1 and high IgG2a Ab responses as well as and $CD4^+$ T cell-derived IFN-γ responses were induced by mucosal (i.e., nasal, oral, and intratracheal) immunization with *H. influenzae* membranes and nCT as adjuvant (62).

We now report that the mucosal adjuvant activity of CT-A/LT-B results in plasma Ab responses characterized by the presence of Ag-specific IgG2a with IgG1 and IgG2b Abs. This pattern of IgG subclass response is similar to that induced by nLT and strongly

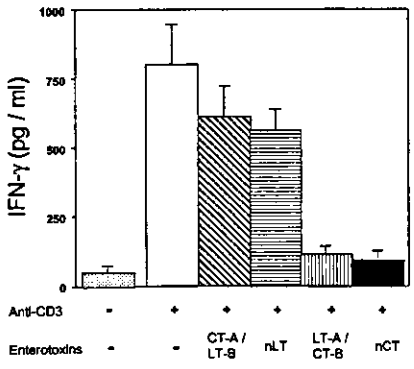

FIGURE 7. The nCT and LT-A/CT-B, but not nLT or CT-A/LT-B, inhibited IFN-γ production by anti-CD3 mAb-stimulated T cells. Naive splenic T cells were cultured for 3 days in the presence of anti-CD3 (1 μg/ml) after no pretreatment (□) or 1 h of pretreatment with 100 ng/ml of CT-A/LT-B (▨), nLT (▤), LT-A/CT-B (▥), or nCT (■). Culture supernatants were subjected to IFN-γ-specific ELISA. Data are expressed as the mean ± 1 SE and are representative of four separate experiments.

suggests a major involvement of the LT-B subunit in this response profile. Interestingly, a completely different pattern of IgG subclass responses, characterized by IgG1 and IgG2b Abs with IgE Ab responses, was seen after nasal immunization with the LT-A/CT-B chimera. This latter profile of Ab responses mimicked those induced by nCT. These results support a major role for the CT-B subunit in Th2-type adjuvanticity. Taken together, the present findings and our recent study with the nontoxic CT-A-E112K/LT-B chimera (42) clearly indicate that the major changes associated with substitution of the B subunit of one enterotoxin with the reciprocal molecule from the other involve Th cell cytokine responses and the associated pattern of IgG subclass and IgE Ab responses.

It is now well accepted that specific cytokines produced by Th cell subsets control the pattern of Ig isotype and IgG subclass Ab responses (31, 63). In this study we confirmed these biased patterns of plasma Ab responses induced by CT-A/LT-B and LT-A/CT-B by analyzing cytokine production by Ag-specific $CD4^+$ T cells. Consistent with the well-established role of IFN-γ for enhancing IgG2a responses in mice (31, 32, 63), in vitro restimulated, OVA-specific $CD4^+$ T cells from mice nasally immunized with OVA and CT-A/LT-B or nLT as adjuvants produced high levels of IFN-γ. On the other hand, elevated levels of IL-4, but not IFN-γ, were seen in culture supernatants from OVA-specific $CD4^+$ T cells isolated from mice given OVA and LT-A/CT-B or nCT as mucosal adjuvants. Both IFN-γ and IL-4 are major cytokines that down-regulate the expression of the opposite Th cell phenotype (32, 64). In fact, IFN-γ production by Th1 cells down-regulates IL-4, a major cytokine produced by $CD4^+$ Th2 cells, and conversely, IL-4 effectively diminishes IFN-γ production by $CD4^+$ Th1 cells (32, 64). Thus, high levels of IL-4 produced by $CD4^+$ T cells from mice that received LT-A/CT-B or nCT most likely resulted in inhibitory signals for $CD4^+$ Th1 cells, while IgG1/IgG2b subclasses and IgE Ab responses would be enhanced. On the other hand, a potent IFN-γ environment elicited by CT-A/LT-B or nLT would inhibit IL-4 production by Th2 cells and would support the development of plasma IgG2a responses. Taken together, our results clearly indicate that biased Th2- or Th1-type responses are induced by LT-A/CT-B or CT-A/LT-B, respectively. We have also provided direct evidence that nCT and LT-A/CT-B down-regulate IL-12R expression on T cells, an effect consistent with a biased Th2 cell subset response.

Since mucosal Ag delivery is critical for the induction of mucosal and systemic immunity, considerable efforts have been dedicated to the development of safe adjuvants for mucosal vaccines that promote host immunity (2, 11, 12, 65). The newly developed nontoxic derivatives of bacterial enterotoxins represent a significant step toward the incorporation of these powerful adjuvants in

human vaccines. In this regard better protection against intracellular pathogens or extracellular Ags and toxins would require the ability to target Th1- or Th2-type responses. The results reported here show that such targeted immune responses could be achieved by chimeric CT/LT molecules. The efficacy of chimeras as adjuvants when administered by the oral route remains to be determined. Since CT-A2 appears to more efficiently stabilize interactions between the A and B subunits than LT-A2 (49), one cannot exclude that the CT-A/LT-B molecules would display adjuvanticity at lower concentrations than nLT when given by the oral route. This possibility is currently under investigation. The CT-B conjugated to an Ag was recently shown to more effectively enhance Ag-specific immunity than did LT-B-Ag conjugates after oral administration (23). The mechanism underlying this difference in adjuvant activity is still unknown. Our chimeric CT/LT molecules could represent unique probes to investigate the mechanisms underlying the mucosal adjuvant activity of these enterotoxins and for the development of mucosal vaccines that induce either Th1- or Th2-type responses.

In summary, we have shown that chimeric molecules made by spontaneous association of the A subunit of CT or LT with the B subunit of the corresponding toxin are both effective mucosal adjuvants for protein vaccines that elicit a pattern of Th cell responses dictated by the origin of the B subunit. Our results clearly show that biased Th1- or Th2-type responses can be elicited by CT/LT chimeras and depend upon the presence of the LT-B or the CT-B subunit, respectively. Since the enterotoxicity of CT and LT can be eliminated by single amino acid substitutions in the A subunit, the development of chimeras composed of the mutant A subunit and either CT-B or LT-B may lead to safe adjuvants for Th1- or Th2-type responses and may thus be suitable for use in human mucosal vaccines.

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Protective Mucosal Immunity in Aging Is Associated with Functional CD4$^+$ T Cells in Nasopharyngeal-Associated Lymphoreticular Tissue[1]

Yukari Hagiwara,* Jerry R. McGhee,* Keiko Fujihashi,* Ryoki Kobayashi,* Naoto Yoshino,* Kosuke Kataoka,* Yuri Etani,* Mi-Na Kweon,† Shinichi Tamura,‡ Takeshi Kurata,§ Yoshifumi Takeda,‖ Hiroshi Kiyono,*†¶ and Kohtaro Fujihashi[2]*

Our previous studies showed that mucosal immunity was impaired in 1-year-old mice that had been orally immunized with OVA and native cholera toxin (nCT) as mucosal adjuvant. In this study, we queried whether similar immune dysregulation was also present in mucosal compartments of mice immunized by the nasal route. Both 1-year-old and young adult mice were immunized weekly with three nasal doses of OVA and nCT or with a nontoxic chimeric enterotoxin (mutant cholera toxin-A E112K/B subunit of native labile toxin) from *Brevibacillus choshinensis*. Elevated levels of OVA-specific IgG Abs in plasma and secretory IgA Abs in mucosal secretions (nasal washes, saliva, and fecal extracts) were noted in both young adult and 1-year-old mice given nCT or chimeric enterotoxin as mucosal adjuvants. Significant levels of OVA-specific CD4$^+$ T cell proliferative and OVA-induced Th1- and Th2-type cytokine responses were noted in cervical lymph nodes and spleen of 1-year-old mice. In this regard, CD4$^+$, CD45RB$^+$ T cells were detected in greater numbers in the nasopharyngeal-associated lymphoreticular tissues of 1-year-old mice than of young adult mice, but the same did not hold true for Peyer's patches or spleen. One-year-old mice given nasal tetanus toxoid plus the chimeric toxin as adjuvant were protected from lethal challenge with tetanus toxin. This result reinforced our findings that age-associated immune alterations occur first in gut-associated lymphoreticular tissues, and thus nasal delivery of vaccines for nasopharyngeal-associated lymphoreticular tissue-based mucosal immunity offers an attractive possibility to protect the elderly. *The Journal of Immunology*, 2003, 170: 1754–1762.

The cellular and molecular mechanisms of immunosenescence have been extensively investigated in systemic lymphoid compartments. For example, it has been suggested that altered T cell functions are major factors in dysregulated immune responses that commonly occur in the elderly (1–7). These include alterations in T cell phenotypes, reduced IL-2 production and IL-2R expression, aberrant signal transduction, and enhanced programmed cell death of naive T cells (1–4). Furthermore, reduced responses to mitogens and impaired cytokine production have also been reported in the elderly (5–7). It has been shown that these alterations closely parallel increases in memory type and loss of the naive T cell phenotype during aging (1, 4, 8). Dysfunctions in B cells and Ab responses also occur in aging (7, 8). For example, recent studies showed that pre-B cells develop poorly in the bone marrow of aged BALB/c mice (8).

Although age-associated changes in the mucosal immune system are less well understood when compared with immunosenescence in systemic immunity, it has been shown that the mucosal immune system is also altered by aging because the elderly are much more susceptible to infections of the gastrointestinal (GI)[3] tract (9, 10). Furthermore, marked increases in the severity and mortality caused by respiratory pathogens such as influenza virus and the bacterial pathogen *Streptococcus pneumoniae* are seen in the elderly (11–13). Additionally, it was reported that the number of lymphocytes in Peyer's patches and mesenteric lymph nodes (LNs) decreased in aged rats (14). Our recent study showed that Ag-specific mucosal and systemic immune responses were diminished in 1-year-old mice immunized orally with OVA and native cholera toxin (nCT), whereas significant immune responses were seen in orally immunized, young adult mice in both mucosal and systemic lymphoid compartments (15). Thus, these studies clearly indicate that the development of age-associated alterations occur earlier in the GI tract mucosa than in systemic immune compartments (15).

Recent studies support the notion that nasopharyngeal-associated lymphoreticular tissue (NALT) is a major mucosal inductive site. To understand the precise contribution of NALT in the induction of IgA responses to inhaled Ags, previous studies have

*Departments of Oral Biology and Microbiology, Immunobiology Vaccine Center, University of Alabama, Birmingham, AL 35294; Departments of †Mucosal Immunology and ‡Translation Research, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan; §Department of Pathology, National Institute for Infectious Diseases, Tokyo, Japan; ¶Division of Mucosal Immunology, Department of Microbiology and Immunology, Institute for Medical Sciences, University of Tokyo, Tokyo, Japan; and ‖Jissen Women's College, Tokyo, Japan

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[2] Address correspondence and reprint requests to Dr. Kohtaro Fujihashi, Department of Oral Biology, Immunobiology Vaccine Center, University of Alabama, 845 19th Street South BBRB, Room 761, Birmingham, AL 35294-2170. E-mail address: kohtarof@uab.edu

[3] Abbreviations used in this paper: GI, gastrointestinal; AFC, Ab-forming cell; CLN, cervical lymph node; GALT, gut-associated lymphoreticular tissue; LT, lymphotoxin; LT-B, B subunit of native labile toxin; mCT, mutant cholera toxin; NALT, nasopharyngeal-associated lymphoreticular tissue; nCT, native cholera toxin; NP, nasal passage; S-IgA, secretory IgA; sIg, surface Ig; SMG, submandibular gland; TT, tetanus toxoid.

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isolated and characterized lymphoid cells from NALT of mice and rats (16–19). In these species, NALT consists of bilateral strips of nonencapsulated lymphoid tissues underlying the epithelium on the ventral aspect of the posterior nasal tract and exhibits a bell-type shape in cross sections (20). Like gut-associated lymphoreticular tissues (GALT), M cells are present in NALT for Ag uptake (21, 22). Thus, M cell-targeted immunization with a reovirus sigma-1 protein coupled to Ag effectively induced Ag-specific immune responses (23). Although dense aggregates of lymphocytes have been observed in the NALT of normal mice, germinal centers are absent, but could be induced by nasal application of Ag (18). Thus, uncommitted B cells ($sIgM^+$) have been found in high proportions (80–85%), while low numbers of $sIgA^+$ and $sIgG^+$ B cells (3–4% and 0–1%, respectively) have been noted in mononuclear cells isolated from NALT (17, 19). Approximately 30–40% of NALT are $CD3^+$ T cells with a CD4:CD8 ratio of ~3.0 (17, 19). The majority of NALT $CD3^+$ T cells coexpress CD45RB, and thus are naive, resting T cells (17, 19). Because transcriptional single cell analysis revealed the expression of mRNA for both Th1 and Th2 cytokines, the majority of $CD4^+$ T cells are considered to be of the undifferentiated Th0 type (2). Furthermore, stimulation via the TCR-CD3 complex resulted in development of both Th1- and Th2-type cells in NALT.

Most current nasal immunization studies instill vaccine into each nostril (usually 5–10 μl/nostril), and normal inhalation subsequently results in effective delivery of vaccine, presumably into NALT. nCT and mutants of CT (mCTs) are effective mucosal adjuvants and have been widely used for nasal immunization with protein Ags, bacterial components, viruses, or virus-related peptides for the induction of secretory IgA (S-IgA) Ab responses and/or protection (24–28). Nasal immunization with OVA and mCT as adjuvant resulted in S-IgA anti-OVA Ab responses in various mucosal tissues (24). Furthermore, mice nasally immunized with pnuemococcal surface protein A Ag plus mCT revealed pnuemococcal surface protein A-specific IgA Ab responses associated with effective protection against capsular serotype 3 *S. pneumoniae* A66 (25). These Ag-specific S-IgA Ab responses were associated with polarized Th2-type responses in cervical LNs (CLN) (24, 25).

In this study, we have examined whether nasal vaccines are effective in aged mice. Our results indicate that nasal immunization with the weak Ag OVA or with the vaccine tetanus toxoid (TT) plus nCT or mCT-A E112K/B subunit of native labile toxin (LT-B) is an effective vaccine approach to induce protective immune responses in aged mice. Furthermore, significant numbers of naive $CD4^+$ T cells were noted in NALT, but not in GALT of 1-year-old mice, and this subset was associated with the induction of protective immunity.

Materials and Methods

Mice

Young adult (6- to 8-wk-old) C57BL/6 and BALB/c mice were purchased from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD). The retired C57BL/6 and BALB/c male breeders (8 mo old) were obtained from The Jackson Laboratory (Bar Harbor, ME). Upon arrival, all mice were immediately transferred to microisolators, maintained in horizontal laminar flow cabinets, and provided sterile food and water ad libitum. Experiments were performed using young adult C57BL/6 and BALB/c mice between 6 and 8 wk of age or aged mice either between 12 and 14 mo (~1 year), or those over 2 years of age. The health of the mice was tested semiannually, and mice of all ages used in these experiments were free of bacterial and viral pathogens.

Chimeric mCT-A E112K/LT-B

A plasmid containing both the mCT-A E112K and LT-B genes was constructed, as previously described (29). Briefly, the mCT-A E112K gene was amplified by PCR using pUC119-E112K as a template, and the PCR product was inserted into the *NcoI*/*Hin*dIII site of $pNCMO_2$ ($pNCMO_2$-mCT-A E112K). The LT-B gene was also amplified by PCR using the chromosome of enterotoxigenic *Escherichia coli* WT-1. The PCR product was cloned into the *NcoI*/*Bam*HI site of $pNCMO_2$ ($pNCMO_2$-LT-B). Next, the mCT-A E112K gene together with the preceding ribosome binding site (SD2) of *Brevibacillus choshinensis* cell wall protein gene was amplified using the BAMSD primer; 5′-AAA GGA TCC TAG AGG AGG AGA ACA CAA GG-3′ and mCT-A-R primer from $pNCMO_2$-mCT-A E112K. The amplified SD2-mCT-A E112K gene was digested and then cloned into the *Bam*HI/*Hin*dIII site of $pNCMO_2$-LT-B, resulting in $pNCMO_2$-LT-B-mCT-A E112K (29). The expression plasmid, $pNCMO_2$-LT-B-mCT-A E112K, was introduced into *B. choshinensis* HPD31 by electroporation. The *B. choshinensis* HPD31 vector containing the plasmid $pNCMO_2$ gene was cultured in 2SLN medium at 30°C for 3 days (29). After cultivation, the mCT-A E112K/LT-B in the culture supernatant was purified using a D-galactose immobilized column (Pierce, Rockford, IL), as described previously (29, 30).

Nasal immunization and sample collection

Aged and young adult mice were immunized three times at weekly intervals with nasal doses of 100 μg of OVA (fraction V; Sigma-Aldrich, St. Louis, MO) and 0.5 μg of nCT (List Biological Laboratories, Campbell, CA) or 5 μg of mCT-A E112K/LT-B in PBS (24, 25, 29). To compare mucosal immune responses in aged mice immunized by different routes, animals were given oral doses of 1 mg of OVA and 10 μg of nCT (15). In tetanus toxin challenge experiments, groups of mice were immunized with TT (25 μg) and mCT-A E112K/LT-B or nCT. TT was kindly provided by Y. Higashi from The Biken Foundation, Osaka University (Osaka, Japan). Plasma and mucosal secretions (nasal washes, saliva, and fecal extracts) were collected on day 21. Saliva was obtained from mice following i.p. injection with 100 μg of sterile pilocarpine (31). Fecal pellets (100 mg) were suspended into 1 ml of PBS containing 0.1% sodium azide and were then extracted by vortexing for 5 min. The samples were spun at 10,000 × *g* for 5 min, and the supernatants were collected as fecal extracts (24, 25, 31). The mice were sacrificed 7 days after the last immunization. The nasal washes were obtained by injecting 1 ml of PBS on three occasions into the posterior opening of the nasopharynx with a hypodermic needle (32).

Ab assays

Ab titers in plasma and mucosal secretions were determined by an ELISA (15, 24, 25, 30). Falcon microtest assay plates (BD Biosciences, Oxnard, CA) were coated with an optimal concentration of OVA (100 μl of 1 mg/ml) in PBS overnight at 4°C. Two-fold serial dilutions of samples were added after blocking with 1% BSA. To detect Ag-specific Ab levels, HRP-conjugated, goat anti-mouse μ, γ, or α, H chain-specific Abs were used (Southern Biotechnology Associates, Birmingham, AL). For IgG Ab subclass determinations, biotinylated mAbs specific for IgG1 and IgG2a (BD PharMingen, San Diego, CA) and peroxidase-conjugated goat anti-biotin Ab were used. End point titers were expressed as the last dilution yielding an OD_{414} of >0.1 U above negative control values after a 15-min incubation.

Enumeration of Ab-forming cells

The spleens and CLNs were removed aseptically, and single cell suspensions were prepared, as described elsewhere (15, 24, 25, 29). For isolation of mononuclear cells from NALT and nasal passages (NPs), a modified dissociation method was used based upon a previously described protocol (17–19). Individual NALT were carefully removed using microsurgical tweezers under a stereoscopic microscope. Following the removal of NALT, the NP tissues were also removed from the nasal cavity. Cells from individual tissues were prepared by gently teasing them through sterile stainless steel screens, followed by enzymatic dissociation using collagenase type IV (0.5 mg/ml; Sigma) to obtain single cell preparations (17–19). Mononuclear cells were purified on discontinuous Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden). Mononuclear cells in the interface between the 40 and 75% layers were removed, washed, and resuspended in RPMI 1640 (Cellgro Mediatech, Washington, DC) supplemented with HEPES buffer (15 mM), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FCS (complete medium). An ELISPOT assay was used to detect cells producing IgM, IgG, and IgA Abs (15, 24, 25, 29). Ninety-six-well nitrocellulose plates (Millititer HA; Millipore, Bedford, MA) were coated with 1 mg/ml solution of OVA for analysis of anti-OVA-specific Ab-forming cells (AFCs) (24, 29, 31).

OVA-specific CD4$^+$ T cell responses

CD4$^+$ T cells were purified by the magnetic-activated cell sorter system (Miltenyl Biotec, Auburn, CA), as described previously (15, 24). Briefly, isolated cells were incubated in a nylon wool column (Polysciences, Warrington, PA) to remove B cells and macrophages. Enriched T cell fractions were then incubated with biotinylated anti-CD4 (GK 1.5) mAb, followed by streptavidin-conjugated microbeads, and passed through a magnetized column. The purified T cell fractions were >97% CD4$^+$ and were >99% viable. Cells were resuspended in complete medium, and purified CD4$^+$ T cells (4×10^6 cells/ml) were cultured with or without 1 mg/ml of OVA in the presence of T cell-depleted, irradiated (3000 rad) splenic APCs. These APCs were derived from naive mice and were placed in 96- or 24-well tissue culture plates (Corning Glass Works, Corning, NY) for 5 days at 37°C in a moist atmosphere of 5% CO_2 in air. To assess OVA-specific T cell responses, 0.5 μCi of tritiated [^{3}H]TdR (Amersham, Arlington Heights, IL) was added for the final 18 h of incubation. The cells were harvested, and the degree of [^{3}H]TdR incorporation was determined by scintillation counting. In some experiments, culture supernatants were harvested after 2 or 5 days of incubation, and were then subjected to cytokine-specific ELISA.

Cytokine-specific ELISA

Levels of cytokines in culture supernatants were measured by ELISA. The details of the ELISA for IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-10 have been described previously (9, 10, 28, 30). For coating and detection, the following mAbs were used: for anti-IFN-γ, R4-6A2 and XMG 1.2 mAbs; for anti-IL-2, JES6-1A12 and JES6-5H4 mAbs; for anti-IL-4, BVD4-1D11 and BVD6-24G2 mAbs; for anti-IL-5, TRFK-5 and TRFK-4 mAbs; for anti-IL-6, MP5-20F3 and MP5-32C11 mAbs; and for anti-IL-10, JES5-2A5 and JES5-16E3 mAbs. The levels of Ag-specific cytokine production were calculated by subtracting the results of control cultures (e.g., without OVA stimulation) from those of OVA-stimulated T cell cultures. This ELISA was capable of detecting 0.78 ng/ml IFN-γ; 8 pg/ml IL-2; 23.4 pg/ml IL-4; 0.78 pg/ml IL-5; 200 pg/ml IL-6; and 0.4 ng/ml IL-10. CD4$^+$ T cells stimulated with a combination of anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) mAb produced 20 ± 5 ng/ml IFN-γ; 40 ± 8 ng/ml IL-2; 120 ± 12 pg/ml IL-4; 1850 ± 150 pg IL-5; 1850 ± 150 pg IL-6; and 20 ± 4 ng/ml IL-10.

Flow cytometry analysis

To assess the frequencies of naive and memory cells in various tissues from young adult and aged mice, aliquots of mononuclear cells ($0.2 - 1 \times 10^6$) from NALT, spleen, and Peyer's patches were isolated from young adult and 1-year-old mice. To obtain single cell preparations, Peyer's patches were dissociated using collagenase type IV (0.5 mg/ml; Sigma-Aldrich) after being carefully excised from the small intestinal wall (31). Mononuclear cells ($0.2 - 1 \times 10^6$) from individual tissues were washed with PBS containing 1% BSA (PBS-BSA). Cells were stained with FITC-conjugated anti-mouse CD4 mAb (GK 1.5; BD PharMingen), PE-labeled anti-mouse CD45RB (23G1; BD PharMingen), and biotinylated anti-mouse CD44 (Pgp-1; BD PharMingen) mAb at 4°C for 30 min. Cells were washed with PBS-BSA and incubated with CyChrome-streptavidin at 4°C for 30 min. These samples were subjected to flow cytometry (FACSCalibur; BD Biosciences) for cell subset analysis.

Tetanus toxin challenge

Tetanus toxin for the challenge experiment was kindly provided by Y. Higashi (The Biken Foundation). The toxin was diluted in 0.5% gelatin/PBS, and an appropriate minimum lethal dose (130 LD_{50}) was given s.c. to each mouse. Individual mice were monitored daily for paralysis and death (33).

Statistics

The data are expressed as the mean ± SEM, and mouse groups were compared with control mice using a Mann Whitney *U* test with Statview II software (Abacus Concepts, Berkeley, CA) designed for Macintosh computers. A p value of <0.05 was considered significant.

Results

Normal OVA-specific IgA Ab responses occur in 1-year-old mice immunized nasally

We first examined OVA-specific mucosal immune responses in 1-year-old mice given nasal OVA plus nCT, because our previous study had shown that impaired mucosal immunity was seen in mice of this age given OVA and nCT by the oral route (15). The young adult and 1-year-old mice were given nasal OVA and nCT as adjuvant, and OVA-specific Ab responses in nasal washes, saliva, and fecal extracts were examined. Significant S-IgA Ab responses occurred in mucosal secretions of both young adult and aged mice (Fig. 1, Table I). Thus, OVA-specific Ab levels in 1-year-old mice were comparable to those seen in young adult mice (Fig. 1, Table I). Similarly, high levels of plasma IgG and mucosal S-IgA Ab responses were induced in BALB/c mice given nasal OVA and nCT (Table I). In contrast, 1-year-old mice given oral OVA and nCT showed significantly reduced Ab responses in plasma and fecal extracts (Table I). These results show that mucosal immune responses after nasal immunization are distinct from those that occur following oral immunization.

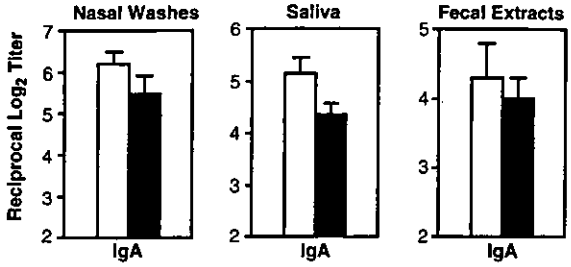

FIGURE 1. Comparison of OVA-specific IgA Ab responses in nasal washes, fecal extracts, and saliva of 1-year-old (filled bars) and young adult (open bars) mice. Each mouse group was nasally immunized once per week for 3 consecutive wk with 100 μg of OVA plus 0.5 μg of nCT as mucosal adjuvant. Seven days after the last nasal immunization, IgA Ab levels in nasal washes, fecal extracts, and saliva were determined by OVA-specific ELISA. The values shown are the mean ± SEM for 15 young adult and 25 1-year-old mice.

OVA-specific plasma Ab and AFC responses in 1-year-old mice

Because it has been shown that both oral and nasal immunization induce Ag-specific immune responses in systemic as well as mucosal sites, OVA-specific plasma Ab levels were assessed. Significant and comparable OVA-specific plasma IgA Ab responses were seen in 1-year-old mice following nasal immunization when compared with young adult mice (Fig. 2). Levels of OVA-specific IgG Abs were elevated in the plasma of young adult mice (Fig. 2). Similarly, 1-year-old mice given nasal OVA plus nCT exhibited OVA-specific, systemic IgG Ab responses that were slightly lower than those seen in young adult mice (Fig. 2). Furthermore, 1-year-old mice given nasal OVA plus nCT showed reduced, but levels still comparable to plasma IgG1 Ab responses of young adult mice (Fig. 2). As expected, low OVA-specific IgG2a Ab responses were

Table I. *Comparison of OVA-specific Ab responses in C57BL/6 and BALB/c mice induced by different immunization routes*[a]

		Reciprocal Log_2 Titer[c]		
		Nasal		Oral
Anti-OVA Ab	Age of Mice	C57BL/6	BALB/c	C57BL/6
IgA[b] (Fecal extracts)	6–8 wk	4.3 ± 0.5	4.6 ± 0.2	5.2 ± 0.7
	12–14 mo	4.0 ± 0.5	4.5 ± 0.3	<2
IgG[b] (Plasma)	6–8 wk	19.3 ± 0.2	19.0 ± 0.4	18.0 ± 1.0
	12–14 mo	17.2 ± 0.3	17.2 ± 0.1	11.5 ± 0.2

[a] C57BL/6 or BALB/c mice were immunized with OVA (nasal, 100 μg; oral, 1 mg) with nCT (nasal, 0.5 μg; oral, 10 μg) three times at weekly intervals.
[b] Plasma and fecal extract samples were collected at day 21 and subjected to OVA-specific ELISA.
[c] Values represent the mean end point titer ± SEM for 10–15 mice in each experimental group.

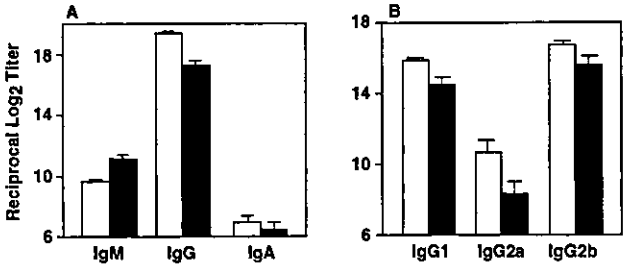

FIGURE 2. Comparison of OVA-specific IgM, IgG, IgA, and IgG subclass Ab responses in plasma of 1-year-old (filled bars) and young adult (open bars) mice. Each mouse group was nasally immunized once per week for 3 consecutive wk with 100 μg of OVA plus 0.5 μg of nCT as mucosal adjuvant. Seven days after the last nasal immunization, IgM, IgG, and IgA (*A*), and IgG subclass (*B*) Ab levels in plasma were determined by OVA-specific ELISA. The values shown are the mean ± SEM for 15 mice in each experimental group.

seen in young adult as well as in 1-year-old mice, and this was due to the propensity of nCT to induce Th2-type responses when used as mucosal adjuvant (24, 25). These results indicate that 1-year-old mice nasally immunized with protein Ag plus adjuvant possess comparable levels of Ag-specific IgG and IgA Ab responses in plasma and in mucosal secretions as those seen in young adult mice. In addition, similar anti-CT-B Ab responses were seen in 1-year-old and young adult mice (data not shown).

Mononuclear cells from NP, submandibular glands (SMG), CLN, and spleen taken from nasally immunized young adult or from 1-year-old mice were subjected to an OVA-specific ELISPOT assay to determine the numbers and isotypes of AFCs present. One-year-old mice exhibit comparable numbers of OVA-specific AFCs in IgA effector sites such as NPs and SMG (Fig. 3). Similarly, 1-year-old mice given OVA and nCT as nasal adjuvant showed intact AFC responses in both spleen and CLNs. Furthermore, 1-year-old mice showed increased numbers of OVA-specific IgM AFCs in spleen, confirming results that this isotype increases in mice as they age (Fig. 3). These results confirm that mucosal immunity mediated through the NALT immune system is maintained in 1-year-old mice. The findings are remarkably different from our previous studies that showed impaired mucosal Ab responses in 1-year-old mice given oral OVA plus nCT (15).

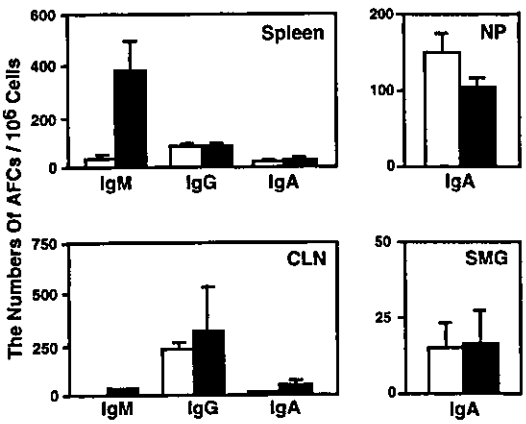

FIGURE 3. Analysis of OVA-specific AFCs in 1-year-old (filled bars) and young adult (open bars) mice immunized nasally with OVA and nCT. Seven days after the last nasal immunization, mononuclear cells isolated from NP, SMG, CLN, and spleen were examined using an OVA-specific ELISPOT assay to determine the numbers of IgM, IgG, and IgA AFCs. The results represent the mean values ± SEM for 15 young adult and 25 1-year-old mice.

T cell proliferative and cytokine responses in 1-year-old mice

Our findings to this point suggest that early, age-associated changes do not occur in the NALT immune system. To examine the roles of nasally induced $CD4^+$ T cells in the induction of mucosal immunity, we next assessed OVA-specific T cell proliferative responses in nasally immunized, young adult and aged mice. The $CD4^+$ T cells from spleen or CLNs were cultured with OVA in the presence of irradiated, T cell-depleted spleen cells. Similar level of splenic $CD4^+$ T cell proliferative responses were seen in young adult and 1-year-old mice (Fig. 4). The $CD4^+$ T cells from CLNs of 1-year-old mice immunized nasally with OVA plus nCT showed lower (but significant (stimulation index = 15)) proliferative responses than did those of young adult mice (Fig. 4). These results clearly support our observations that the NALT-mediated immune responses in 1-year-old mice are intact and suggest that mucosal age-associated alterations have not taken place in the NALT immune system.

We next examined Ag-specific Th1 and Th2 cytokine responses in young adult and 1-year-old mice. The culture supernatants harvested from OVA-stimulated $CD4^+$ T cells isolated from spleen and CLNs of 1-year-old mice given nasal OVA plus nCT exhibited high levels of OVA-induced IL-4, IL-5, IL-6, and IL-10 responses (Fig. 5). The levels of these Th2-type cytokine responses were almost identical with those of young adult mice and ~20–50% of the cytokine synthesis induced by anti-CD3 and anti-CD28 mAb-triggered, splenic $CD4^+$ T cells. In contrast, only low levels of OVA-induced IFN-γ and IL-2 production were detected in the $CD4^+$ T cell cultures from spleen and CLNs of both young adult and aged mice when compared with anti-CD3 and anti-CD28 mAb-stimulated cultures (Fig. 5). These results indicate that Th2-type cytokines, especially IL-4, mediate the adjuvant effects of nCT and were maintained in 1-year-old mice when this adjuvant was given by the nasal route, although mice of this age failed to respond to oral OVA plus nCT as adjuvant (15). Taken together, our results indicate that when mice are immunized via the NALT immune system, OVA-specific, $CD4^+$ T cell responses escape the influence of age-associated dysfunction that occurs in 1-year-old mice immunized via GALT.

Nasal immunization induces plasma, but not mucosal Abs in 2-year-old mice

To this point, our findings indicate that nasal immunization effectively induces both mucosal and systemic immune responses in 1-year-old mice. Thus, it was important to examine whether these

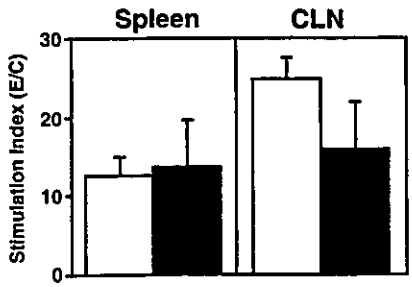

FIGURE 4. Analysis of Ag-specific $CD4^+$ T cell proliferative responses induced by nasal immunization with OVA plus nCT. $CD4^+$ T cells from spleen and CLN of 1-year-old (filled bars) and young adult (open bars) mice were cultured with or without OVA in the presence of APCs for 5 days. The stimulation index was determined as cpm of wells with OVA/wells without OVA (controls). The level of [^{3}H]TdR incorporation for each control well was between 500 and 1000 cpm. The results represent the individual values from three separate experiments.

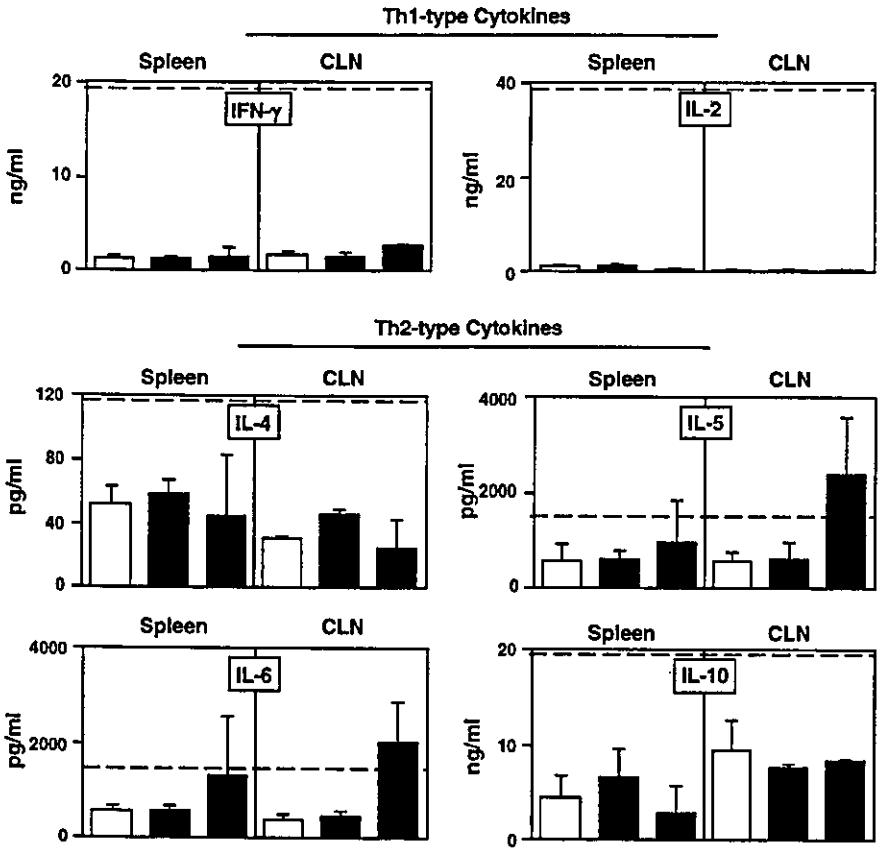

FIGURE 5. OVA-induced, $CD4^+$ Th1- and Th2-type cytokine responses in young adult (open bars) and aged (1-year-old (▨) or 2-year-old (filled bars)) mice given nasal OVA plus nCT. The CLN or splenic $CD4^+$ T cells (4×10^6 cells/ml) from each mouse group were cultured with 1 mg/ml of OVA in the presence of T cell-depleted and irradiated splenic feeder cells (8×10^6 cells/ml). Culture supernatants were harvested after 5 days of incubation (or 2 days for IL-2) and analyzed by the respective cytokine-specific ELISA. The scale shows maximum production of each cytokine when $CD4^+$ T cells were stimulated with anti-CD3 and anti-CD28 mAbs. In the case of IL-5 and IL-6 production, the maximum protein levels were 1700–2000 pg/ml. The values shown are the mean ± SEM of 15 mice in each experimental group.

responses also occur in severely aged mice. Two-year-old mice were immunized nasally with OVA plus nCT three times at weekly intervals, and mucosal secretions and plasma were collected 1 wk after the final immunization. Significant reductions in OVA-specific, S-IgA Ab responses were noted in all mucosal secretions (nasal washes, saliva, and fecal extracts) of 2-year-old mice given nCT nasally when compared with identically immunized, young adult or 1-year-old mice (Fig. 6, $p < 0.05$). Conversely, plasma IgG and IgG subclass anti-OVA Ab levels in 2-year-old mice that received nasal nCT as adjuvant were significant and were comparable to those of young adult and 1-year-old mice (Fig. 6).

Specific OVA-induced Th2-type cytokine responses supported the presence of these OVA-specific Ab responses in the systemic compartment of 2-year-old mice because essentially the same levels of IL-4, IL-5, and IL-6 synthesis by OVA-stimulated splenic $CD4^+$ T cells were noted (Fig. 5). These results suggest that although mucosal immunosenescence is delayed in the NALT immune system when compared with GALT, age-associated alterations initially take place in mucosal compartments before they occur in systemic lymphoid tissues.

Analysis of NALT T cell subsets in aging

To explore the mechanism for mucosal immune responses in 1-year-old mice given nasal vaccine, the frequencies of naive $CD4^+$ T cells, which are responsible for initiation of immune responses, were compared with those in young adult mice. Mononuclear cells from NALT, Peyer's patches, and spleen of nonimmunized mice were stained with FITC-conjugated anti-CD4, PE-labeled anti-CD45RB, and biotinylated anti-CD44 mAbs, followed by CyChrome-streptavidin, and were then subjected to flow cytometry analysis. The naive $CD4^+$ T cell subset in spleen, Peyer's patches, and NALT of 1-year-old mice was significantly reduced when compared with those cells in young adult mice. In addition, the actual number of mononuclear cells in spleen and Peyer's patches was also reduced; however, the total number of cells in NALT doubled in 1-year-old mice (Table II). Thus, no overall reductions in the actual numbers of $CD4^+$, $CD45RB^+$ T cells were noted in NALT of 1-year-old mice (Table II). These results indicate that

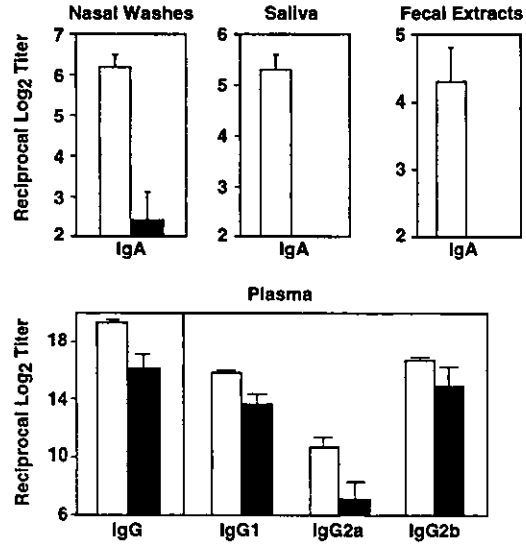

FIGURE 6. Comparison of OVA-specific IgA and IgG Ab responses in nasal washes, fecal extracts, saliva, and plasma of 2-year-old (filled bars) and young adult (open bars) mice. Each mouse group was nasally immunized weekly for 3 consecutive wk with 100 μg of OVA plus 0.5 μg of nCT as mucosal adjuvant. Seven days later, IgA Ab levels in nasal washes, fecal extracts, saliva, and IgG and IgG subclass Ab responses in plasma were determined by OVA-specific ELISA. The values shown are the mean ± SEM of 15 mice in each experimental group.