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(2) Nontoxic CT (E112K) Mutated In The COOH-Terminal KDEL Of The A Subunit Elicits Mucosal Adjuvant Activity Without Intracellular Trafficking, **Y.Hagiwara**, T.Dohi, Y.Kawamura, N.Yoshino, K.Kataoka, K.Komase, Y.Suzuki, H.Kiyono, J.R.McGhee and K.Fujihashi 4th World Congress on Vaccines and Immunization, Tsukuba Science City, Japan, 2004/9/30-10/3

(3) Nontoxic Mutant CT (E112K/KDGL) Elicits Antigen-specific CD8+ Cytotoxic T Lymphocyte (CTL) Responses , **Y.Hagiwara**, A.Hino, K.Kataoka, K.Komase, Y.Suzuki, H.Kiyono, J.R.McGhee and K.Fujihashi, 第34 回日本免疫学会総会・学術集会, 札幌, 2004/12/1-3

H.知的所有権の取得状況

1. 特許取得 なし
2. 実用新案登録 なし
3. その他 なし

研究成果の刊行に関する一覧

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研究成果の刊行物・別刷

A Novel Adjuvant for Mucosal Immunity to HIV-1 gp120 in Nonhuman Primates[1]

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The development of a safe and effective mucosal adjuvant is a crucial step toward a mucosal HIV/AIDS vaccine. This study seeks to determine the promise of a nontoxic mutant of cholera toxin (mCT; E112K) as a mucosal adjuvant in nonhuman primates. HIV-1 gp120 was nasally administered together with mCT E112K or native CT (nCT) as adjuvant on five to six occasions over a 6- to 8-wk period to groups of four rhesus macaques and alone to two monkeys that acted as controls. Macaques given nasal gp120 with either mCT E112K or nCT showed elevated gp120-specific IgG and IgA Ab responses with virus-neutralizing activity in both their plasma and mucosal external secretions, as well as higher numbers of gp120-specific IgA Ab-forming cells in their mucosal and peripheral lymphoid tissues and of IL-4-producing Th2-type CD4-positive ($CD4^+$) T cells than did controls. Even though significant mucosal adjuvanticity was seen with both mCT E112K and nCT, neuronal damage was observed only in the nCT-treated, but not in the control or mCT E112K-treated groups. These results clearly show that mCT E112K is an effective and safe mucosal adjuvant for the development of a nasal HIV/AIDS vaccine. *The Journal of Immunology,* 2004, 173: 6850–6857.

It is well known that HIV-1 infections occur through contact with contaminated blood or during unprotected vaginal or anal intercourse. Indeed, it is estimated that 70–85% of HIV-1 infections are transmitted sexually (1–3). Given that fact, immune responses at mucosal surfaces in which the virus crosses the epithelium of the genital or rectal tracts are an essential component of vaccine-induced protection. The evidence for an association between mucosal immune responses and protection in humans has stemmed from studies on the immune system of women who remained seronegative despite a high rate of exposure to HIV-1. High levels of secretory IgA were detected in the genital secretions of the protected women (4–7). Because the mucosa of the small and large intestine are the largest source of lymphocytes and APCs in the host (8, 9), they act as a potential reservoir for HIV-1-infected cells in viral pathogenesis (10). Studies to develop a HIV/AIDS mucosal vaccine have been conducted in nonhuman primate (NHP)[4] models by using recombinant SIV proteins or peptides (11–17), live-attenuated SIVs (18–23), SIV-encoded virus or bacterial vectors (24–29), DNA vaccines (30–33), and a prime/boost regimen (34–36). Collectively, these studies point to the importance of a mucosal HIV/AIDS vaccine for the prevention of HIV-1 infection.

Recent studies have shown that nasal immunization is the most effective approach for the induction of both mucosal and systemic immune responses (37). For example, nasal immunization with protein/peptide vaccines together with mucosal adjuvant more effectively induces mucosal immunity in the female reproductive tract than does oral immunization (38). Like its gut-associated lymphoreticular tissue counterpart in the gastrointestinal tract, the nasopharyngeal-associated lymphoreticular tissue-based immune system is key to the induction of Ag-specific mucosal and systemic immune responses (39–41). In this regard, we have shown that nasal immunization of rhesus macaques with SIV $p55^{gag}$ together with native cholera toxin (nCT) as mucosal adjuvant induced $p55^{gag}$-specific IgA and IgG Ab responses in vaginal secretions (16).

Although a potent mucosal adjuvant, nCT is not practical for use in humans because of its toxicity. Nasal application of CT B subunit (CT-B) or nCT resulted in its accumulation in the olfactory bulbs of the CNS through GM1 binding and in its subsequent retrograde axonal transport into the olfactory neurons (39). Furthermore, nCT is known to induce high levels of total and Ag-specific IgE Ab responses due to the nature of IL-4-dependent adjuvanticity (40–43). To overcome these potent pathological problems of nCT, we have developed and characterized two nontoxic mutants of cholera toxin (mCT; E112K and S61F) that retain adjuvant properties despite lacking the ADP-ribosyltransferase enzyme activity associated with toxicity (42, 43). Studies by our own group and by others have shown that mutant CT E112K is one of the most effective, safe, and stable adjuvants among the toxin-based mutants that have been tested (41–43).

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[4] Abbreviations used in this paper: NHP, nonhuman primate; AFC, Ab-forming cell; LP, lamina propria; mCT, nontoxic mutant of cholera toxin; MLN, mesenteric lymph node; nCT, native cholera toxin; NGF, nerve growth factor; NP, nasal passage; SMG, submandibular gland.

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Because HIV-1 is most often transmitted via mucosal surfaces, a mucosal vaccine capable of inducing protective Abs and/or CTLs in mucosal tissues and external secretions would act as a first line of defense at the site of initial invasion. We take the first step toward the ultimate goal of developing a safe and effective mucosal adjuvant for a mucsoal HIV/AIDS vaccine in humans by assessing in this study the efficacy and safety of mCT E112K as a mucosal adjuvant in nonhuman primates.

Materials and Methods

HIV-1 immunogen and adjuvant used

HIV-1_{LAI} *Env* gp120 was kindly provided by Quality Biologicals (Gaithersburg, MD) through Contract N01-AI 65278 of the Vaccine Research and Development Branch, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. *Escherichia coli* strains containing the plasmids for the mCT E112K were grown in Luria-Bertani medium (10 mg/ml NaCl, 5 mg/ml yeast extract, 10 mg/ml tryptone) with 100 μg/ml ampicillin (42, 43). The mCT E112K was purified using a D-galactose-immobilized column (Pierce, Rockford, IL) from a cell suspension prepared by sonication of the recombinant *E. coli*, as described previously (42, 43). The purity of mCT E112K was assessed by SDS-PAGE, and no contaminating proteins were noted. The nCT was purchased from List Biological Laboratories (Campbell, CA).

Rhesus macaques

Five mature female and seven male rhesus macaques (*Macaca mulatta*), bred in captivity and reproductively cycling, were obtained from the California Regional Primate Research Center (Davis, CA). They were confirmed negative for Abs to HIV-2, SIV, type D retrovirus, and simian T cell lymphotropic virus-1 (STLV-1), and were maintained in conditions that fully complied with the standards of the American Association of Accreditation of Laboratory Animal Care at the California Regional Primate Research Center.

Immunization methods and schedule used

Rhesus macaques were divided into four groups and nasally immunized with vaccine containing: 1) 100 μg of gp120 alone, 2) 100 μg of gp120 plus 10 μg of nCT, 3) 100 μg of gp120 plus 25 μg of mCT E112K, or 4) 100 μg of gp120 plus 100 μg of mCT E112K. Macaques were anesthetized with ketamine and placed in dorsal recumbancy with head tilted back so that the nares were pointed upward (16). Vaccine solution (0.5 ml) was instilled dropwise into each nostril without inserting the syringe into the nasal cavity. Macaques were kept in that position for 10 min and then placed in lateral recumbancy until they recovered from anesthesia, as described previously (16). Nasal immunization was conducted on days 0, 7, 14, 28, 42, and 56.

Collection of peripheral blood, tissues, and external secretion samples and lymphocyte isolation

Tissues and peripheral blood were harvested using sterile techniques, and appropriate biohazard precautions were observed. The PBMCs were isolated from heparinized peripheral blood using Lymphocyte-Mammal (Cedarlane Laboratories, Hornby, Canada) (44). Plasma, vaginal washes consisting of a mixture of cervical and vaginal secretions, rectal washes, nasal washes, and saliva were collected, as previously described (16). These four external secretions along with the plasma were stored at −80°C until used for the analysis of gp120-specific Ab responses. For isolation of lymphocytes from different mucosal tissues, a modified enzymatic dissociation procedure was used (15, 16). Nasal passages (NP) and submandibular glands (SMG) were dissociated using collagenase type IV (0.5 mg/ml; Sigma-Aldrich, St. Louis, MO) in RPMI 1640 (Mediatech, Washington, DC) for 30 min at 37°C. After removal of Peyer's patches, the small intestine was treated first with PBS containing 1 mM DTT and then with 1 mM EDTA, while lamina propria (LP) mononuclear cells were isolated using the same method as for the NPs. The lymphocytes from tissues were purified using a discontinuous 40 and 75% Percoll gradient (Amersham Biosciences, Piscataway, NJ), as described previously (15, 16).

Monoclonal Abs

The mAbs used for cell surface staining in flow cytometric analysis were as follows: FITC-, PE-, or PerCP-conjugated mAb to human CD3 (SP34; BD Biosciences, San Jose, CA), CD4 (SK3; BD Biosciences), and CD8 (SK1; BD Biosciences). Cross-reactivity of these mAbs for the rhesus macaque was determined using the method described previously (45). However, the observed cross-reactivity with IL-5, IL-10, and IL-13 is a new finding and has not been published previously.

HIV-1 env *gp120-specific ELISA and ELISPOT assays*

HIV-1 *env* gp120-specific IgG, IgM, and IgA Ab titers in plasma, saliva, nasal washes, as well as rectal and vaginal lavages were determined by ELISA, as described previously (15, 16). The HIV-1 *env* gp120-specific IgG, IgM, and IgA Ab-forming cells (AFCs) were also determined by ELISPOT assay, as described elsewhere (15, 16).

Cytokine-specific ELISPOT assay

The PBMCs or lymphoid cells from various tissues were cultured in 10% FCS containing RPMI 1640 (Mediatech) supplemented with HEPES buffer (10 mM), L-glutamine (2 mM), nonessential amino acid solution (10 ml/L), sodium pyruvate (10 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and gentamicin (80 μg/ml) (complete medium) with or without 5 μg/ml HIV-1 *env* gp120, 1 μg/ml anti-human CD28 (CD28.2; BD Biosciences), and anti-human CD49d (9F10; BD Biosciences) mAbs at 37°C with 5% CO_2. Nonadherent cells were harvested after 3 days of incubation and stained with anti-human CD3 and CD8 mAbs. The FACSVantage (BD Biosciences) was used to sort out a subset of $CD3^+CD8^-$ T cells. The frequencies of $CD4^+$ Th1- and Th2-type cytokine-producing cells were determined by using rhesus macaque cytokine-specific ELISPOT kits (U-CyTech, Utrecht, The Netherlands).

In vitro HIV-1 neutralization assay

The diluted plasma or appropriate mucosal secretion was heat inactivated (56°C for 30 min) and incubated with 20 $TCID_{50}$ (50% tissue culture infective dose) units of HIV-1_{LAI} overnight at 4°C. This mixture was then cocultured with 1×10^6 M8166 cells for 2 h (16, 46, 47). After being washed twice with PBS, the cells were cultured in complete medium for 4 days at 37°C. Following incubation, culture supernatants were subjected to Lumipulse (chemiluminescence enzyme immunoassay/full automatic analyzer; Fujirebio, Tokyo, Japan) for measurement of HIV p24. The results were expressed as the percent inhibition of p24 *gag* production in culture supernatants when compared with the cultures containing pre- or nonimmunized plasma or mucosal secretions (16, 46, 47).

Nerve growth factor-β1 (NGF-β1) production in macaque olfactory tissues

The nasal turbinate region of the olfactory tissues was obtained from each macaque at the time of sacrifice. At the termination of the study, the nasal turbinate was perfused with PBS at 25°C. This was followed by perfusion with 100 ml of Zamboni's fixative (4% paraformaldehyde, 15% picric acid) in 0.1 M phosphate buffer. The olfactory bulbs and tubinates were removed and placed in fresh 4% paraformaldehyde at 4°C overnight. The tissue was then transferred to a 30% sucrose solution at 4°C for 48 h to cryoprotect it before sectioning. The tissue was then frozen in OCT compound, and the frozen sections (6 μm) were placed on precoated microscope slides (10% BSA in saline). For staining of sections, all slides were pretreated with rabbit IgG Ab to block nonspecific binding, followed by a biotinylated rabbit anti-human NGF-β1 Ab (Chemicon International, Temecula, CA) used at a concentration of 2 μg. The Ab-stained sections were incubated at 4°C overnight. The slides were then rinsed in three changes of PBS for 2 min and then reacted with avidin-biotin conjugate for 30 min at 25°C. The tissues were rinsed three times with PBS, and then reacted with 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA) for 5–10 min before being again rinsed three times and having sections counterstained with hematoxylin for 30 s. After being washed in distilled water, the slides were dehydrated in 100% alcohol and xylene. In some experiments, the anti-NGF-β1 Ab-stained sections were incubated with HRP-conjugated streptavidin-Alexa Fluor 488 (Molecular Probes, Eugene, OR). Sections were examined with a fluorescence microscope (BX50/BXFLA; Olympus, Tokyo, Japan) equipped with a digital image capture system (Olympus).

Statistics

The results are expressed as the mean ± SEM. Immunized NHP groups were compared with the controls using a Mann-Whitney *U* test with Statview II software (Abacus Concepts, Berkeley, CA) designed for Macintosh computers. A p value of <0.05 or less was considered significant.

Results

Plasma anti-gp120-specific Ab responses

In this study, we have assessed the mucosal adjuvanticity of mCT E112K in rhesus macaques nasally immunized with HIV-1 gp120. Eleven macaques were given 100 μg of gp120 by the nasal route. In addition to the gp120, five macaques were given two doses of mCT E112K as nasal adjuvant, two (Rh09 and Rh91) receiving a 25 μg dose and three (NHPs Rh16, Rh39, and Rh85) receiving a 100 μg dose. As a positive control, and because our previous research showed that nCT is a potent nasal adjuvant for NHPs (16), four other macaques (Rh07, Rh35, Rh60, and Rh88) were given 10 μg of nCT along with gp120. As a negative control, the two remaining macaques (Rh43 and Rh51) were given gp120 alone. The gp120-specific IgG and IgA Abs in plasma of individual macaques were sequentially assessed by an endpoint ELISA. As expected based upon our previous studies (16), significant levels of gp120-specific IgG Ab responses were detected in plasma of all macaques given gp120 with nCT (Fig. 1; $p < 0.01$). Interestingly, comparable gp120-specific IgG Ab responses were observed in macaques receiving 100 μg of mCT E112K as nasal adjuvant (Fig. 1; $p < 0.01$), while much lower levels of these responses were noted in macaques receiving 25 μg of mCT E112K as nasal adjuvant ($p > 0.1$). Furthermore, the group receiving 100 μg of mCT E112K showed comparable gp120-specific plasma IgA Ab responses to those receiving nCT as mucosal adjuvant. In contrast, the two macaques given gp120 alone or those receiving only 25 μg of mCT E112K showed low to undetectable IgA Ab responses. When gp120-specific plasma Ab responses were compared between the two groups given 25 or 100 μg of mCT E112K groups, the group given the higher dose showed greater IgG ($p < 0.01$) and IgA Ab responses than did the group given 25 μg of mCT E112K. Taken together, these results show that 100 μg of mCT E112K is an appropriate dose for inducing HIV-1 gp120-specific plasma Ab responses.

Induction of gp120-specific mucosal immune responses

The gp120-specific IgA and IgG Ab titers were assessed in the mucosal secretions (saliva; nasal, vaginal, and rectal lavages) of macaques given nasal gp120 and mCT. The peak titers of IgG and IgA Abs occurred 7 or 14 days after the last nasal immunization

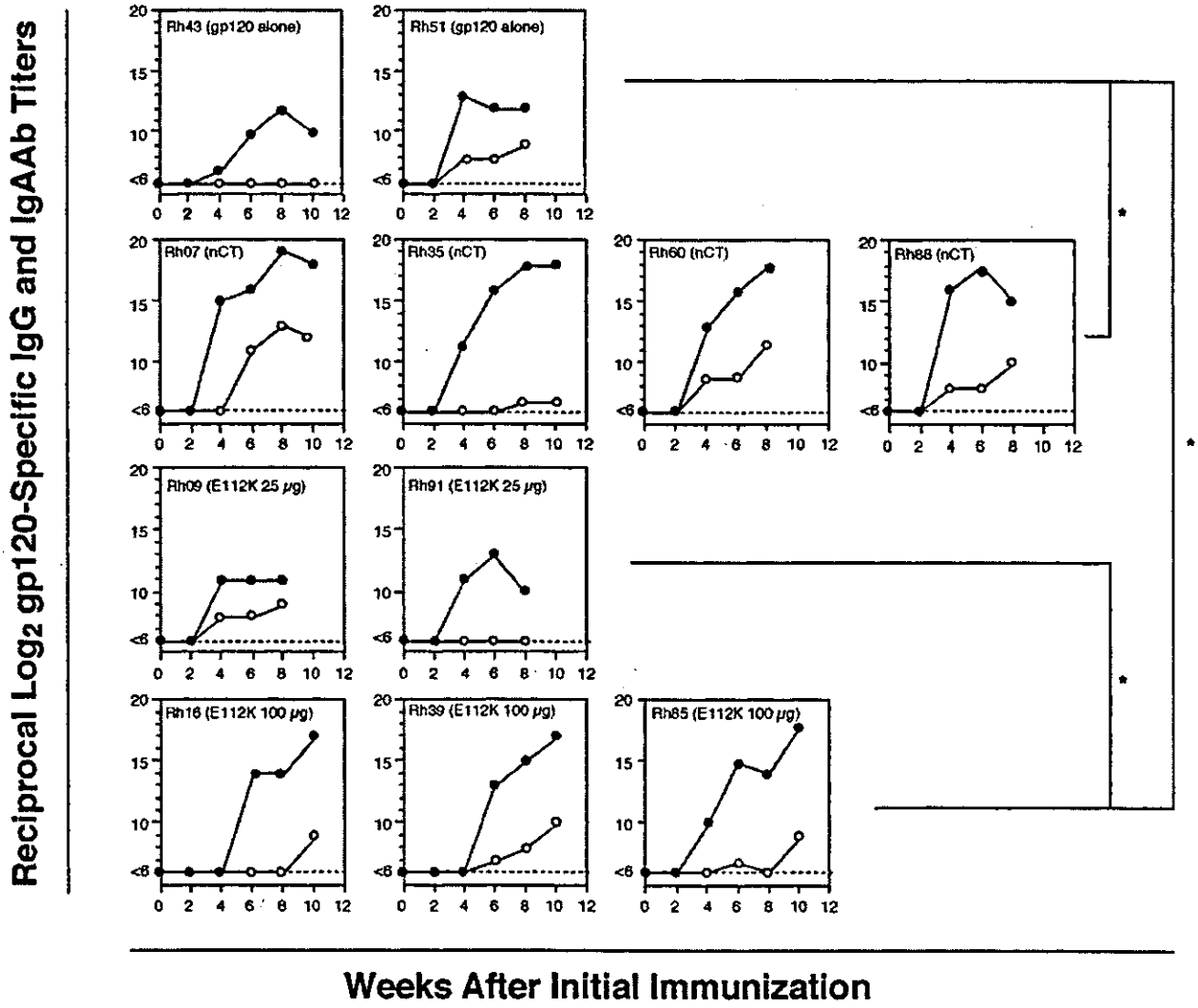

FIGURE 1. HIV-1 gp120-specific plasma IgG (●) and IgA (○) Ab titers were determined by endpoint ELISA. Rhesus macaques were nasally immunized with 100 μg of gp120 alone (Rh43 and Rh51), 100 μg of gp120 and 10 μg of nCT (Rh07, Rh35, Rh60, and Rh88), 100 μg of gp120 and 25 μg of mCT E112K (Rh09 and Rh91), or 100 μg of gp120 and 100 μg of mCT E112K (Rh16, Rh39, and Rh85). The data shown are endpoint titers for each macaque. *, $p < 0.01$ for IgG titers.

Table I. *gp120-specific Ab responses in mucosal secretions of rhesus macaques given a nasal vaccine*

Nasally Immunized with		Identification Number of Macaque	Anti-gp120-Specific Reciprocal Log_2 Ab Titers[a]							
			Saliva		Nasal washes		Vaginal washes		Rectal washes	
gp120	Adjuvant		IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG
100 (μg)	E112K (25 μg)	Rh09	<1[b]	2	NA[c]	NA	<1	3	NA	NA
		Rh91	2	2	NA	NA	<1	<1	NA	NA
	E112K (100 μg)	Rh16	6	5	5	3	NA	NA	3	<1
		Rh39	8	7	5	4	NA	NA	2	3
		Rh85	4	6	4	<1	NA	NA	3	<1
100 (μg)	nCT (10 μg)	Rh60	6	6	NA	NA	2	6	NA	NA
		Rh88	2	<1	NA	NA	<1	4	NA	NA
		Rh07	7	7	7	4	NA	NA	3	<1
		Rh35	5	5	2	<1	NA	NA	<1	<1
100 (μg)	None	Rh51	<1	<1	NA	NA	<1	2	NA	NA
		Rh43	3	2	<1	<1	NA	NA	<1	<1

[a] Saliva, nasal, vaginal, and rectal washes were collected 7 or 14 days after final immunization and were then subjected to gp120-specific ELISA.
[b] Endpoint titers were expressed as the last dilution giving an OD_{460} of 0.1 U above samples obtained from nonimmunized controls.
[c] NA, Not available.

(Table I). The findings for mucosal secretions paralleled those for plasma described above, with a dose of 100 μg of mCT E112K inducing gp120-specific IgA and IgG Ab levels comparable to those seen in macaques receiving nCT, but with a dose of only 25 μg of mCT E112K failing to support induction of gp120-specific Ab responses (Table I). These findings further support the notion that 100 μg of mCT E112K is the optimal dose for nasal adjuvanticity. Furthermore, our results demonstrate that a nasal vaccine of HIV-1 gp120 and mCT E112K as mucosal adjuvant would be an effective regimen for induction of anti-HIV-1 immune responses in external secretions of NHPs.

Induction of gp120-specific AFCs in mucosal lymphoid tissues

The induction of gp120-specific Ab responses was further confirmed at the level of plasma cell AFC responses. Comparable numbers of HIV-1 gp120-specific IgA and IgG AFCs were seen in the nasal passages of macaques immunized with gp120 plus either the optimal dose of mCT E112K (Rh39) or nCT (Rh60). Similarly, the numbers of gp120-specific IgA AFCs in SMGs and intestinal LP of macaques given the optimal dose of mCT E112K were comparable to those seen in positive controls given nCT as mucosal adjuvant (Fig. 2). These findings show that nasally coadministered mCT possesses adjuvant activity for the induction of gp120-specific AFCs in mucosal effector tissues.

gp120-specific CD4$^+$ Th1 and Th2 cell responses

Because nasal mCT showed adjuvant activity in both mucosal and systemic lymphoid compartments, HIV-1 gp120-specific CD4$^+$ Th1- and Th2-type responses were assessed using a cytokine-specific ELISPOT assay. When restimulated with gp120 in vitro, mononuclear cells from spleen and mesenteric lymph nodes (MLNs) of macaques immunized with gp120 and either mCT E112K or nCT induced both Th1 (IFN-γ)- and Th2-type (IL-4, IL-10, and IL-13) cytokine-producing CD4$^+$ T cells (Fig. 3). Both the group given mCT E112K and that given nCT showed higher numbers of IL-4- and IL-13-producing CD4$^+$ T cells in MLNs than those observed in the two macaques nasally immunized with gp120 alone. The nCT-immunized group exhibited higher numbers of IL-4- and IL-13-producing CD4$^+$ T cells than did the mCT E112K-immunized macaques, but the latter group showed higher numbers of IL-10-producing CD4$^+$ T cells were noted in their MLNs. A similar pattern of Th2-type cytokine production was seen in the spleens of these two groups of macaques. The IFN-γ-producing CD4$^+$ T cells were also seen in both MLNs and spleens

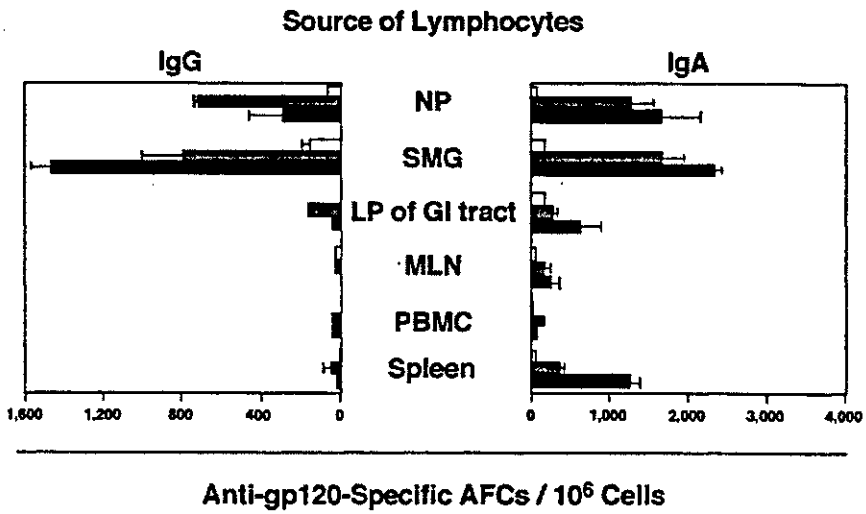

FIGURE 2. The gp120-specific IgG and IgA AFCs in mucosal and systemic lymphoid tissues of rhesus macaques (Rh51, □; Rh60, ▨; Rh39, ■) were determined by ELISPOT assay. Mononuclear cells were isolated from NPs, SMGs, the LP of the gastrointestinal tract; MLNs; spleen; and PBMC 2 wk following the final immunization. The results shown are the mean AFCs/10^6 cells ± SEM.

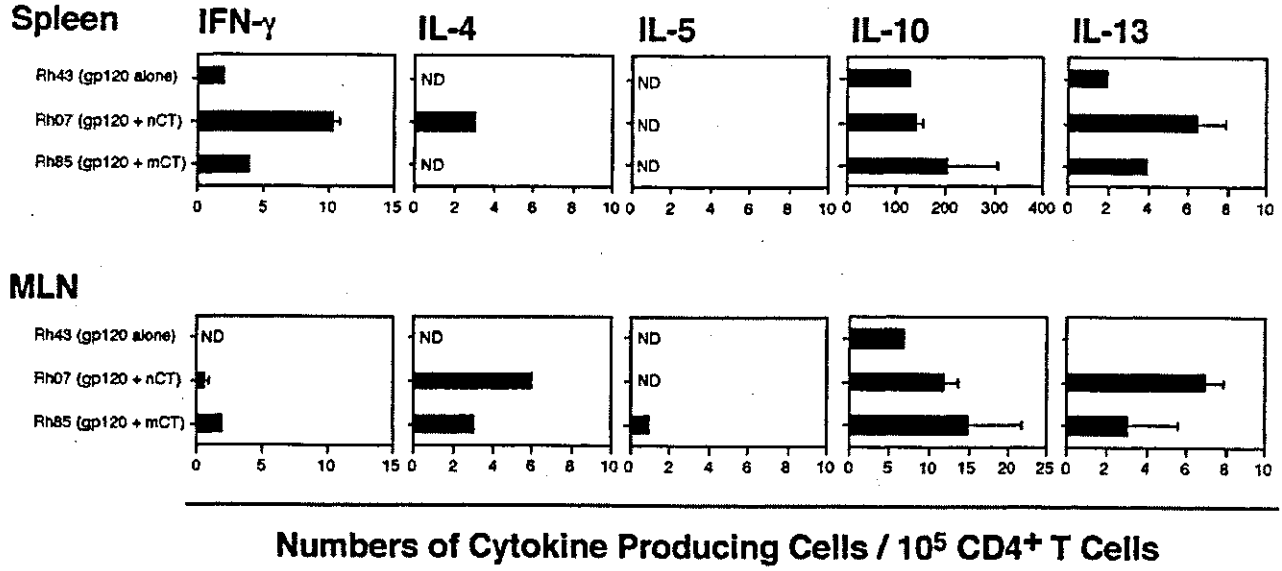

FIGURE 3. Th1 and Th2 cytokine production by gp120-stimulated $CD4^+$ T cells isolated from spleens and MLNs of rhesus macaques (Rh43, Rh07, and Rh85). Lymphocytes were cultured with or without gp120 for 3 days. Nonadherent cells were harvested and stained with anti-human CD3 and CD8 mAbs. A subset of $CD3^+$, $CD8^-$ T cells was purified using flow cytometry. The purified $CD4^+$ T cells were subjected to an array of macaque-specific cytokine (IFN-γ, IL-4, IL-5, IL-10, and IL-13) ELISPOT assays.

of macaques given either mCT E112K or nCT as mucosal adjuvant. Interestingly, the numbers of IFN-γ-producing $CD4^+$ T cells in the MLNs of both groups were lower than those seen in the spleens. These results suggest that mCT as nasal adjuvant preferentially induces Ag-specific Th2-type cytokine-producing $CD4^+$ T cells, while also somewhat enhancing the induction of Th1-type cytokine-producing $CD4^+$ T cells.

$HIV\text{-}1_{LAI}$-neutralizing Abs in external secretions and plasma

It was important to examine whether gp120-specific Abs in external secretions or plasma induced in NHPs given nasal gp120 and mCT E112K as mucosal adjuvant possessed HIV-neutralizing activity. To assess neutralizing activity, we performed an in vitro neutralization assay using $HIV\text{-}1_{LAI}$. The plasma (1/10 dilution) from macaques given nasal gp120 plus mCT E112K showed ~75–90% inhibition of $HIV\text{-}1_{LAI}$, a significantly higher rate than that seen in control plasma samples from either naive macaques or NHPs given gp120 only (Fig. 4*A*). Furthermore, the nasal lavages (1/10 dilution) from two rhesus macaques (Rh16 and Rh85) given nasal gp120 plus mCT E112K exhibited 35 and 55% inhibition of $HIV\text{-}1_{LAI}$, a rate of inhibition comparable to that seen in NHPs given nasal nCT as mucosal adjuvant. In contrast, control groups (naive macaques or those given gp120 alone) possessed little ability to inhibit $HIV\text{-}1_{LAI}$ (<20%) (Fig. 4*B*). These results clearly show that nontoxic mCT E112K can be used as a mucosal adjuvant for the induction of HIV-1-specific neutralizing immunity in both external secretions and plasma.

Safety of mCT E112K when used as a nasal adjuvant in NHPs

To assess the threat of neuronal damage posed by nasal vaccines containing gp120 and mCT E112K, NGF-β1 production in nasal turbinates of olfactory tissues was examined. Macaques given gp120 with nCT exhibited areas of intense NGF-β1 production in the olfactory region, which was associated with neuronal damage

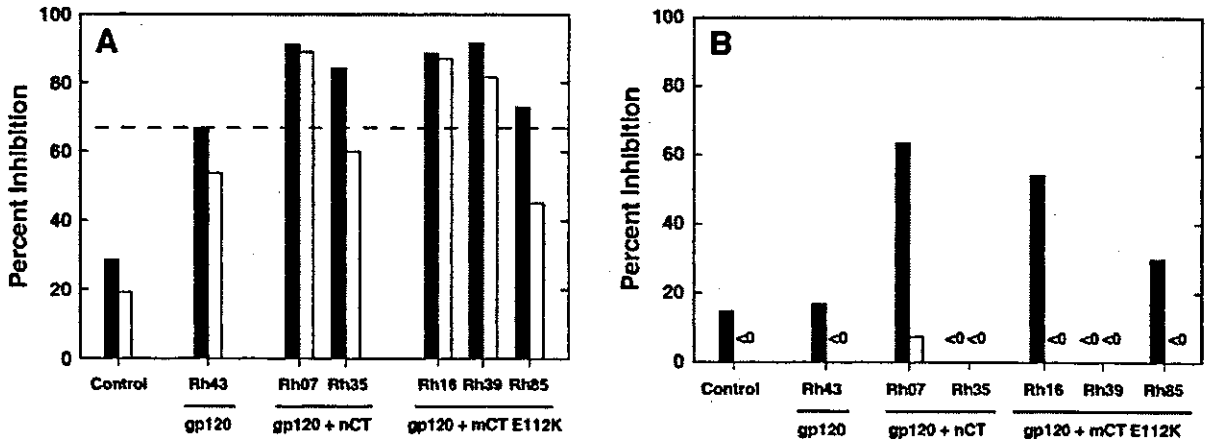

FIGURE 4. In vitro neutralization of HIV-1 was performed with a standard p24 release assay. The plasma (*A*) and nasal wash (*B*) samples were collected 2 wk after the final immunization. Samples were diluted 1/10 (■) or 1/100 (□) and were analyzed for the presence of neutralizing Abs against a homogenous laboratory strain ($HIV\text{-}1_{LAI}$). As controls, plasma samples were obtained from macaques before nasal immunization (preimmune sample). The results are the mean values of three separate assays.

and inhibition of apoptosis (Fig. 5*C*). In contrast, macaques given nasal gp120 plus mCT E112K (Fig. 5*B*) expressed very minimal levels of NGF-β1, which were essentially the same as those seen in olfactory tissues taken from the macaques given nasal gp120 alone (Fig. 5*A*). These results indicate that mCT E112K, although as effective a mucosal adjuvant as nCT, possesses none of its toxicity for neuronal tissues. As a safe and potent mucosal adjuvant, mCT E112K could speed the development of a nasal HIV-1 vaccine in humans.

Discussion

This study clearly provides direct evidence that mCT E112K is an effective mucosal adjuvant for the induction of HIV-1-specific immunity in the NHP model. When used as a nasal adjuvant, mCT E112K induced gp120-specific Abs possessing HIV-neutralizing activity in both external secretions and plasma, but showed negligible toxicity for the CNS-associated tissues of rhesus macaques. In contrast, nCT elicited increases in NGF-β production, a major manifestation of CNS inflammation. Thus, our study is the first to provide evidence establishing the efficacy and safety of an adjuvant for use in higher mammals. Collectively, our findings convincingly demonstrate the potential of mCT E112K as a mucosal adjuvant in humans and suggest that it may be time to take the next step toward the development of nasal vaccines, including those for HIV-1, by beginning clinical trials.

Our previous studies have already shown the efficacy and safety of mCT E112K as a nasal adjuvant in the murine system (43, 47, 48). In our earlier studies, we established that nasal immunization with pneumococcal surface protein A or diphtheria toxoid plus mCT E112K elicited sufficient Ag-specific immune responses to provide protection after lethal challenge with either *Streptococcus pneumoniae* bacteria or diphtheria exotoxin (48, 49). Furthermore, nasal application of mCT together with protein Ags elicited both Ag-specific IgA and IgG Ab responses in mucosal and systemic lymphoid tissue compartments (43, 47, 48). Among the different forms of mutant toxin-based adjuvants, mCT E112K was shown to be the safest and most effective in the murine model (41–43). However, until now, no studies assessing the mucosal adjuvanticity of different forms of toxin-based mutant adjuvants such as our mCT E112K had been performed in a large mammalian animal model, i.e., NHPs. Among the mammalian models, we chose the NHP experimental model as the most appropriate to and useful for the development of an HIV/AIDS mucosal vaccine.

AIDS is well known to be a sexually transmitted disease caused by HIV-1 infection via mucosal surfaces. The NHP experimental model of SIV infection has provided detailed evidence for the mucosal transmission of the virus, and has shown that the inhibition of its entry via the mucosa led to protection against disease development (50). Accordingly, an effective HIV/AIDS vaccine will be more readily developed if the potential of the common mucosal immune system is tapped, because mucosal immunization is known to induce effective protection against pathogens at mucosal surfaces as well as in lymphoid tissue compartments (37, 38, 51). Of note, our previous study showed that nasal immunization with SIV $p55^{gag}$ plus nCT as mucosal adjuvant induced in vaginal secretions of rhesus macaques Ag-specific Ab responses with virus-specific neutralizing Ab activity. In the case of the NHP experimental model, our studies have shown that mucosal (both oral or nasal) immunization with SIV $p55^{gag}$ plus nCT induced Ag-specific humoral and cellular immunity in both mucosal and systemic immune systems of rhesus macaques (15, 16, 52).

Despite its strong mucosal adjuvanticity, nCT is of little practical value as a mucosal adjuvant in humans because of its toxicity. Thus, much effort has been expended on the creation of genetically manipulated nontoxic mutants of CT that would retain adjuvanticity, but not toxicity. In the current study, we sought to examine the mucosal adjuvanticity of mCT E112K as nasal adjuvant when coadministered to rhesus macaques with HIV-1 gp120. In this study, we provide the first evidence that the nasal application of mCT E112K as a mucosal adjuvant effectively induces HIV-1 gp120-specific Ab responses in both mucosal and systemic lymphoid tissues of rhesus macaques. Furthermore, plasma and nasal

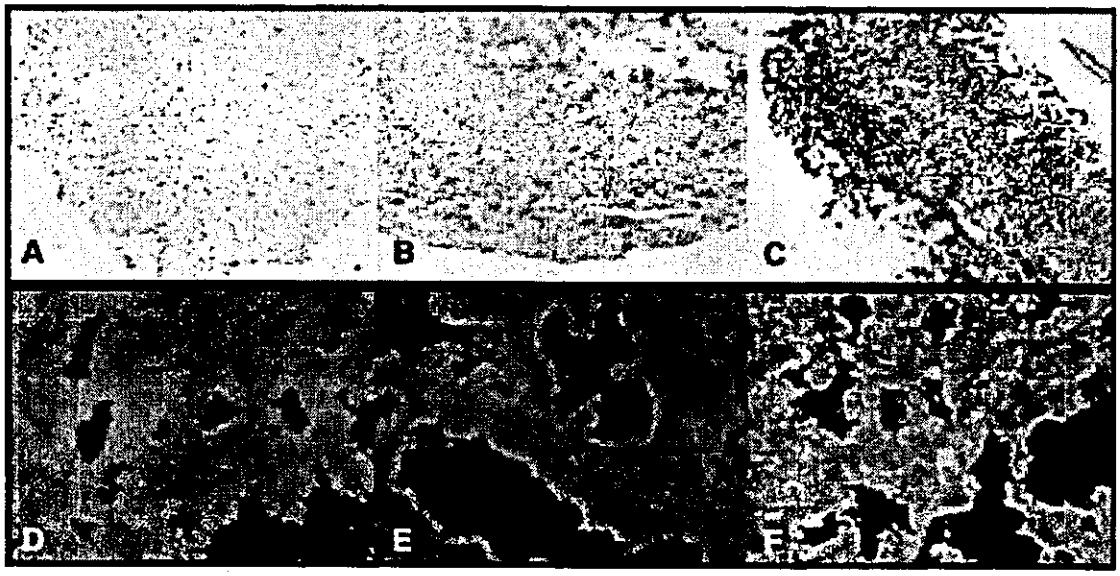

FIGURE 5. Detection of NGF-β1 expression in olfactory bulbs of rhesus macaques nasally immunized with gp120 and either nCT or mCT E112K as mucosal adjuvant. The anti-NGF-β1 Ab-stained sections were reacted with avidin-biotin conjugate, followed by 3,3′-diaminobenzidine (*A–C*), or incubated with HRP-conjugated streptavidin-Alexa Fluor 488 (*D–F*). *C* (×40) and *F* (×100), Show high expression of NGF-β1 along neuronal tracts when rhesus macaques were given nCT and gp120. *B* (×40) and *D* (×100), Show tissues from a macaque given gp120 plus mCT E112K. *A* (×40) and *D* (×100), Illustrate tissues from a macaque given gp120 alone.

washes from macaques given nasal gp120 plus mCT E112K contained HIV-1_{LAI}-neutralizing Abs. These findings clearly demonstrate the efficacy of mCT E112K as a mucosal adjuvant and suggest its potential for use in trial vaccines in humans.

However, nCT and even some of its nontoxic mutant forms pose additional, more specialized dangers when administered via the nasal route, the route of choice for mucosal vaccines because of its efficacy at inducing Ag-specific immune responses. Nasal vaccines using either nCT or one of its nontoxic mutants as adjuvant risk entering the CNS because of the proximity of the olfactory nerves/epithelium and olfactory bulbs to the brain. This potential for neurotoxicity has been a major obstacle for the use of enterotoxin-based mucosal adjuvants, even nontoxic mutant forms, in humans via the nasal route.

Our own studies have shown the potential toxicity of nCT for the olfactory nerves/epithelium and olfactory bulbs (39). Thus, neuronal association of CT-B through GM1 ganglioside binding appears to preclude efficient clearing of these enterotoxin-based mucosal adjuvants and to cause extended accumulation of them in neuronal tissues associated with the olfactory tract (39). These results show that nasally administered CT derivatives retain some toxicity and are targeted to the CNS, posing a serious obstacle to human use. Indeed, recent reports showed that a human vaccine containing inactivated influenza and native labile toxin as an adjuvant resulted in a very high incidence of Bell's palsy (53, 54). These results strongly indicate that it is essential to develop a more safe and effective nasal vaccine for human use.

Our current findings demonstrate the promise of the nontoxic form of mCT E112K as a safe and effective mucosal adjuvant and so point the way to the development of better nasal vaccines. The nontoxic form of mCT E112K did not elicit any increase in NGF-β expression by the olfactory tissues of NHPs. Only minimal NGF-β1 synthesis, comparable to that seen in NHP given nasal gp120 alone, was detected in the olfactory CNS tissues of rhesus macaques given nasal mCT E112K as nasal adjuvant.

Our previous study showed that nasal immunization with $p55^{gag}$ plus nCT induced $p55^{gag}$-specific T cell responses in both mucosal and systemic lymphoid tissue compartments (16). Thus, it was shown that both IFN-γ and IL-2 (Th1-type) expression as well as IL-5, IL-6, and IL-10 (Th2-type) production were seen in Ag-stimulated $CD4^+$ T cells isolated from NHPs given nasal $p55^{gag}$ and nCT. In this regard, our current study has shown that both Th1 (IFN-γ)- and Th2 (IL-10 and IL-13)-type cytokine-producing $CD4^+$ T cells were present in the MLNs and spleens of rhesus macaques given either mCT E112K or nCT as a nasal adjuvant. Although the viral Ags used in the current study are different from those in the previous report, our results also showed that mCT E112K provided adjuvanticity in NHPs through the generation of both Th1- and Th2-type cytokine responses by $CD4^+$ T cells. Induction of IFN-γ-producing $CD4^+$ T cells by nasally coadministered mCT E112K may be an additional benefit because it may lead to the generation of Ag-specific cell-mediated immunity responses. In viral infections including HIV and SIV, CTL activity has been shown to be of central importance for host defense and to correlate well with IFN-γ production (44). In this regard, we postulate that nasally coadministered mCT E112K would also induce CTL activity in various mucosal tissues. Confirming this prediction, rhesus macaques given nasal nCT as mucosal adjuvant showed SIV-specific CTL activity (16). We are currently testing Ag-specific CTL activity in macaques given nasal mCT E112K as mucosal adjuvant.

In conclusion, the current study has provided significant new information for a potential human phase I clinical trial using the nontoxic form of toxin mucosal adjuvant mCT E112K. Thus, nasal immunization of rhesus macaques with gp120 and mCT E112K resulted in the induction of Ab-neutralizing immunity against HIV-1 by inducing gp120-specific IgA and IgG Abs in both mucosal and systemic lymphoid tissue compartments, respectively. Furthermore, the safety of nasal mCT E112K was confirmed by the lack of CNS damage in this NHP model. This important new evidence supports the candidacy of mCT E112K as a potentially important mucosal adjuvant for use in humans.

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Pathological Role of Large Intestinal IL-12p40 for the Induction of Th2-Type Allergic Diarrhea

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IL-12 consists of two disulfide-linked subunits, p40 and p35, that form functionally active heterodimers for the induction of Th1 cells. In contrast to IL-12 heterodimers, p40 monomers and homodimers possess inhibitory effects on Th1 cells leading to the creation of a Th2 environment. Although it has been shown that IL-12p40 acts as antagonist of IL-12p70 *in vitro*, no evidence is currently available whether IL-12p40 is functional *in vivo*. We now report that IL-12p40 plays an important pathological role in an intestinal allergic disease. A high expression of IL-12p40 protein was demonstrated in epithelial cells, dendritic cells, and macrophages in large but not small intestine of allergic diarrhea-induced mice. Interestingly, neutralization with anti-IL-12p40 mAbs reduced the incidence and delayed the onset of disease development. Lower levels of ovalbumin (OVA)-specific IgE Abs in serum were detected in anti-IL-12p40 mAb-treated mice than in control Ab-treated mice. The secretion of Th2 cytokines and eotaxin by the mononuclear cells isolated from the large intestine of anti-IL-12p40 mAb-treated mice was significantly decreased. Finally, the removal of the IL-12p40 gene resulted in complete inhibition of disease development. These results show that over-expression of IL-12p40 is an important contributing factor for the generation of the dominant Th2-type environment in the large intestine of mice with allergic diarrhea. *(Am J Pathol 2004, 164:1327–1335)*

In general, intestinal allergic reactions are provoked by the activation of allergen-specific Th2-type cells, excessive eosinophil and mast cell recruitment, and IgE Ab production.[1,2] We previously reported that systematically primed BALB/c mice developed severe diarrhea after repeated oral challenge with ovalbumin (OVA).[1] Diarrhea-induced mice revealed a Th2-type allergic response characterized by high levels of Ag-specific IgE Abs in serum, increased numbers of IgG1, IgA and IgE Abs in the large intestine, and high numbers of mast cells and eosinophils in the large intestine. Furthermore, large intestinal $CD4^+$ T cells isolated from mice with allergic diarrhea secreted IL-4, IL-5, and IL-13, but not IFN-γ. On the other hand, a murine model of eosinophilic gastrointestinal hypersensitivity induced by challenge with oral allergen, in the form of enteric-coated beads, resulted in marked allergen-induced IL-4 and IL-5 production and eosinophil accumulation in the small intestine. Although several interesting intestinal allergic models were recently reported,[2,3] the exact underlying molecular and cellular mechanisms remain to be elucidated.

In allergic asthma, allergen-specific T cells have been shown to also acquire the Th2 phenotype and to avoid from the Th1-type pathway.[4,5] A recent study has demonstrated that Th1/Th2 imbalance induced allergic disease at the level of transcription factors. Interestingly, a high expression of GATA-3 and/or a lack of T-bet signaling markedly influenced the development of allergic asthma.[6,7] In addition, the expression of not only Th2-type cytokine but also Th1-type cytokine (ie, IFN-γ or IL-12) played a critical role in murine dermatitis and asthma models.[8,9] It has also been demonstrated that natural killer (NK) cells, like Th2 cells, play an important role in the development of allergen-induced asthma.[9]

It has been suggested that antigen-presenting cells (APCs) play a crucial role in the skewing of Th1 and Th2 differentiation.[10,11] IL-12 is a heterodimeric cytokine composed of p40 and p35 which strongly promotes the differentiation of naive $CD4^+$ T cells to the Th1 phenotype and suppresses the synthesis of Th2-type cytokines.[12] IL-12 is produced primarily by APCs and the production is regulated by IL-10 and IFN-γ.[13,14] In addition, biological effects of IL-12 are counter-balanced by IL-12p40 itself, which binds to the receptor complex without induc-

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ing intracellular signals.[15] Thus, murine IL-12p40 inhibits IL-12-mediated responses by means of the competitive binding to IL-12 receptor with an affinity similar to that of IL-12p70.[16,17] Further, IL-12p40 can behave as an IL-12p70 antagonist *in vivo*, delaying the allograft rejection of cardiac myoblast.[18] IL-12p40 transgenic mice also showed increased susceptibility to the malaria infection.[19]

With regard to allergic responses, a potential contribution of IL-12 has been suggested for the development of allergic asthma.[9,20] Mixed Th1- and Th2-associated cytokines, including IFN-γ, IL-2, IL-5, GM-CSF, and IL-12, were secreted by smooth muscle cells located in the sensitized airways of atopic asthma-induced mice.[21] A previous study demonstrated that IL-12p40 mRNA expression was detected in the lung tissue of mice with asthma.[21] However, the role of IL-12 in intestinal allergic disease has not yet been carefully examined. To investigate the potential roles of IL-12 in intestinal hypersensitivity, we have assessed the expression pattern of IL-12p40 or IL-12p35 in the intestinal tract of diarrhea-induced mice and examined the therapeutic effects of modulating IL-12 involvement in allergic diarrhea.

Materials and Methods

Mice

BALB/c mice were purchased from Japan Clea Company (Tokyo, Japan). Breeding pairs of IL-12p40-deficient [IL-12p40 knockout (KO)] mice were purchased from The Jackson Laboratories (Bar Harbor, ME), and colonies were established and maintained in the experimental animal facility at the University of Tokyo and the University of Alabama at Birmingham. All mice were 6 to 7 weeks of age at the beginning of individual experiments.

Antibodies

Recombinant murine IL-12 p40 and p70 were obtained from BD PharMingen (San Diego, CA). Biotin-anti-CD11b (M1/70, rat IgG2b) and biotin-anti-CD11c (HL3, hamster IgG) were also purchased from BD PharMingen. Biotin-SP-conjugated, affinity-purified anti-rat IgG (H+L) mouse $F(ab')_2$ and anti-hamster IgG(H+L) goat $F(ab')_2$ were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Anti-IL-12p40 (C17.8.20, rat IgG2a) was a generous gift from Dr. G. Trinchieri, Wistar Institute, Philadelphia, PA[22] and was purified from ascites on a protein G column (Pharmacia Biotech, Uppsala, Sweden). Purified rat IgG was purchased from Sigma Chemical, Inc. (St. Louis, MO).

Induction of Allergic Diarrhea

For the induction of allergic diarrhea, our well-established protocol was used as described previously.[1] Briefly, on the first day of the experiment (day 0), mice were primed by subcutaneous (SC) injection of 1 mg of OVA in Complete Freund Adjuvant (CFA) (Difco Laboratories, Detroit, MI). One week after the systemic priming (day 7), mice were repeatedly challenged with 50 mg of OVA by oral administration (PO) three times per week for several weeks.[1] These mice were sacrificed and analyzed within 1 and 2 hours after a total of 10 times of oral administration with OVA. In a timed kinetics study, mice were sacrificed at indicated intervals, ie, 0, 30 minutes, 1 hour, and 2 hours following the last oral administration of OVA. As controls, mice were repeatedly given oral OVA in phosphate-buffered saline (PBS) without systemic priming or were injected SC with 1 mg OVA in CFA without repeated oral challenge.

Treatment of Mice with Anti-IL-12p40

In vivo Ab treatment was performed as described previously.[23] Anti-IL-12p40 (C17.8.20, rat IgG2a) was a generous gift from Dr. G. Trinchieri, Schering-Plough Research Institute, Dardilly, France.[22] BALB/c mice were intraperitoneally administered with 0.5 mg to 2.0 mg of purified anti-IL-12p40 (C17.8) or control rat IgG (Sigma Chemicals, Inc.) per week for the duration of the experiment. Ab treatment was started 1 week before or at the time systemic priming with OVA in CFA. Among different concentrations tested, the protocol of 1 mg/mouse of per week was the most optimal condition. Further, when the mAb treatment schedule was compared between the start at 1 week before or at the same time as OVA systemic priming, an identical effect was noted in this study. Thus, the mAb anti-IL-12 (1 mg/mouse) was started on 1 week before the OVA systemic priming.

ELISA for OVA-Specific IgE Abs in Serum

To assess OVA-specific IgE Ab levels in serum, a sandwich ELISA system was adopted.[1] End-point titers of OVA-specific IgE Abs were expressed as the reciprocal $\log_2$ of the last dilution that showed a level of 0.1 higher absorbance than that of sera of non-immune mice as background.

Isolation of Mononuclear Cells and Cytokine-Specific ELISA Assay

To isolate mononuclear cells from small and large intestines, we used a enzymatic dissociation method.[24] Briefly, mononuclear cells were dissociated by collagenase from small and large intestines after the removal of Peyer's patches and were then subjected to a discontinuous Percoll gradient.[25] Mononuclear cells from small and large intestines were then co-cultured in the presence of 1 mg OVA. After 3 days of culture, the supernatant was collected and assayed for cytokines by using an ELISA Kit specific for IL-4 (Endogen, Woburn, MA), IL-5 (Amersham Pharmacia Biotech, Piscataway, NJ), IL-13 (R&D Systems, Minneapolis, MN) and eotaxin (Techne Corporation, Minneapolis, MN).

Table 1. Primers and Probes Used for Qualitative and Quantitative RT-PCR

		Conventional PCR*	
HPRT	primer	F	GTT GGA TAC AGG CCA GAC TTT GTT G
		R	GAG GGT AGG CTG GCC TAT AGG CT
IL-12p40	primer	F	ATG GCC ATG TGG GAG CTG GAG
		R	TTT GGT GCT TCA CAC TTC AGG
IL-12p35	primer	F	ACC CAG TTG GCC AGG GTC
		R	CAA GGC ACA GGG TCA TCA TC
		Real-time PCR†	
HPRT	primer	F	AAC TTT GCT TTC CCT GGT
		R	AGT CAA GGG CAT ATC CAA CA
	probe	FITC	CAG TAC AGC CCC AAA ATG GTT AAG GTTGC
		LCRed640	AGC TTG CTG GTG AAA AGG ACG TCT CG
IL-12p40	primer	F	AGA GGA GGG GTG TAA CCA G
		R	GGG AAC ACA TGC CCA CTT G
	probe	FITC	ACC GAA GTC CAA TGC AAA GGC GG
		LCRed640	AAT GTC TGC GTG CAA GCT CAG G
IL-12p35	primer	F	CCT GTG CCT TGG TAG CAT CT
		R	AGA CTG CAT CAG CTC ATC G
	probe	FITC	ACC AGA CAG AGT TCC AGG CCA TCA
		LCRed640	TGA TGG CCT GGA ACT CTG TCT GGT

*, Primers were designed based on the published sequence.[13]
†, Primers and probes were designed and produced by Nihon Gene Research Laboratories (Sendai, Japan).

Immunoprecipitation and Western Blot Analyses

For the detection of different forms of IL-12, intestinal tissue extracts were prepared as previously described with minor modifications.[26] Small and large intestines were removed, minced in cold PBS with protease inhibitor, homogenized, and incubated to allow cytokine release from the tissue. After centrifugation intestinal tissue extracts were subjected to the measurement of protein concentration and then pre-cleared with protein G Sepharose beads (Pharmacia Biotech, Uppsala, Sweden), subsequently incubated with anti-IL-12p40, mixed with protein G Sepharose beads. The beads were washed, subjected to SDS-PAGE under non-reducing condition. After electrophoresis, proteins were transferred to a polyvinylidene difluoride microporous membrane (PVDF Immobilon; Millipore, Bedford, MA) and the membrane was reacted with biotinylated anti-IL-12 (C17.8) followed by incubation with biotin-streptavidin complex (ABC-AP Kit; Vector Laboratories, Inc.). Visualization of the signal was performed by NBT/BCIP Substrate Kit (BioRad, Hercules, CA).

Analysis for IL-12 mRNA Accumulation

The expression of IL-12p40 or p35 in small and large intestines of mice was examined using conventional RT-PCR as previously described[13] and quantitative real-time PCR method using a Lightcycler (Roche Diagnostics, GmbH Mannheim, Germany), with some modifications.[27,28] The sequences of primers and probes for real-time PCR were designed by Nihon Gene Research Laboratories (Sendai, Japan) (Table 1). Total RNA was extracted by using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and 2 μg of extracted RNA was subjected to RT reaction using Superscript II Reverse Transcriptase (Life Technologies).[14] Hypoxanthine phosphoribosyl transferase (HPRT) cDNA was used to standardize the total RNA content. The cDNA from each experimental sample was then subjected to the Lightcycler FastStart DNA Master Hybridization Probes Kit (Roche Diagnostics). The external standards of HPRT, IL-12p40, and IL-12p35 DNA prepared by PCR between 20 pg to 0.02 fentograms were used for the quantification of specific cDNA in each sample. The ratio of the p40 and p35 increase in experimental mice was calculated and compared with non-treated mice as follows; the ratio = (p40 or p35 mRNA amounts from experimental mice/HPRT mRNA amounts from experimental mice)/(p40 or p35 mRNA amounts from non-treated mice/HPRT mRNA amounts from non-treated mice).

Immunohistochemical Analysis

Following extensive washing, small and large intestines were fixed in 4% paraformaldehyde-PBS and treated with sucrose-gradient, frozen in OCT-embedding medium as previously described with minor modifications.[29] For IL-12p40 immunostaining, cryosections were subjected to antigen retrieval using 10 mmol/L citric buffer pH 6.0 for 5 minutes at 98°C. Slides were then blocked with normal mouse IgG and incubated with rat anti-IL-12p40 or control rat IgG for 16 hours at 4°C. The section were then treated with biotinylated goat anti-rat IgG $F(ab')_2$ (Jackson ImmunoResearch Laboratories, Inc.), ABC-AP Kit, and red chromogen (Vector Red; Vector Laboratories, Inc.). For IL-12p35 immunostaining, we used goat anti-IL-12p35 (Santa Cruz, Inc., Santa Cruz, CA) or control goat IgG. The sections were then treated with biotinylated donkey anti-goat IgG $F(ab')_2$ and ABC-AP Kit. In the case of surface marker staining, serial sections were incubated with anti-CD11b (M1/70, BD PharMingen) or anti-CD11c (HL3, BD PharMingen), biotinylated second anti-

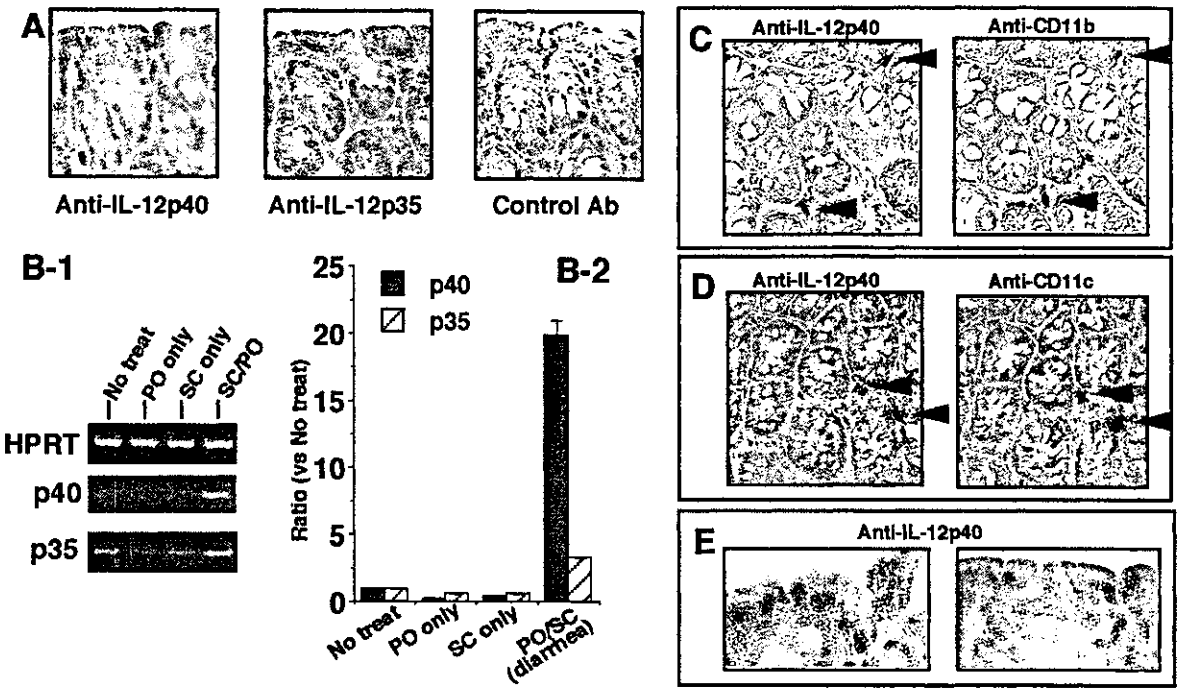

Figure 1. Selective production of IL-12p40 by the large intestine of diarrhea-induced mice. In **A**, large intestinal tissues from diarrhea-induced mice were immunostained with anti-IL-12p40 mAb, anti-IL-12p35 mAb, or control IgG. Control non-disease mice section gave no signal above background (data not shown). In **B-1**, IL-12p40-specific mRNA was expressed selectively in the large intestine of mice with allergic diarrhea. In **B-2**, quantitive real-time PCR analysis of IL-12p40- and p35-specific mRNA expression was performed. The ratio was obtained as the level of IL-12p40 or p35 expression in non-treated mice as a scale of one. The detailed information for the expression of this ratio is described in the Materials and Methods section. In **C-E**, IL-12p40 was detected in MØ and DC and epithelial cells in the large intestine. The serial sections of the large intestine from diarrhea-induced mice were stained with anti-IL-12p40 mAb and anti-CD11b mAb (**C**), with anti-IL-12p40 mAb and anti-CD11c mAb (**D**). The **arrows** point to double-positive cells. Large intestinal epithelial cells were stained with anti-IL-12p40 mAb (**E**).

body and ABC-AP. The color reaction was developed using Vector Red Substrate Kit I.

Statistical Analysis

Statistical analyses were performed by the two sample non-parametric Welch test with a significance level of 0.01 (**) for body weight and Ig levels, respectively. Mouse disease rates were determined using the Wilcoxon rank-sum test with a significance level of 0.01 (**). Values for cytokine-synthesis in the samples between anti-IL-12 p40-treated and control antibody-treated mice were analyzed by using Student's *t*-test at *P* values of <0.01(**).

Results

Detection of IL-12p40 Protein in the Large Intestine of Allergic Diarrhea Mice

To examine whether IL-12p40 was expressed in the large intestine of OVA-induced diarrhea mice, we analyzed IL-12 expression using a variety of available detection methods. First, we performed immunohistochemical analysis to directly demonstrate the enhanced IL-12p40 expression in the large intestine of mice with allergic diarrhea. As shown in Figure 1A, IL-12p40, but not IL-12p35, was expressed in the large intestine of diarrhea-induced mice. To further confirm enhanced expression of IL-12p40 in the large intestine of mice with diarrhea, we next performed IL-12-specific RT-PCR analysis. Interestingly, IL-12p40 mRNA was only detected in the large intestine of diarrhea-induced mice, not in control mice without the disease [eg, SC only or per oral challenge (PO) only; Figure 1B]. In contrast, IL-12p35 mRNA expression was detected in both groups of mice (Figure 1B-1). When IL-12-specific mRNA quantitative real-time PCR analysis was performed, high levels of IL-12p40-specific mRNA were noted in the large intestine of OVA-induced allergic diarrhea mice (Figure 1B-2). In contrast, the level of p35 did not vary among the four different groups including experimental diseased (SC/PO) and control non-diseased mice (non-treated, SC only, and PO only). Taken together, these results clearly indicate that IL-12p40, but not p35, was selectively enhanced at the levels of both mRNA and protein in the large intestine of allergic diarrhea mice.

Inasmuch as the induction of IL-12p40 selectively occurred in the large intestine of OVA-induced allergic diarrhea mice, it was important to determine which cell types produced IL-12p40 in the large intestine. Immunohistochemical analysis demonstrated that IL-12p40-producing cells were co-stained with anti-CD11b mAb [ie, macrophages (MØ)]. Further, $CD11c^+$ cells [ie, dendritic cells (DC)] were also positively stained for IL-12p40 (Figure 1, C and D). Further, some epithelial cells were also positive for IL-12p40 expression (Figure 1E). Taken together, these findings show that large intestinal macrophages, dendritic cells, and epithelial cells are responsi-