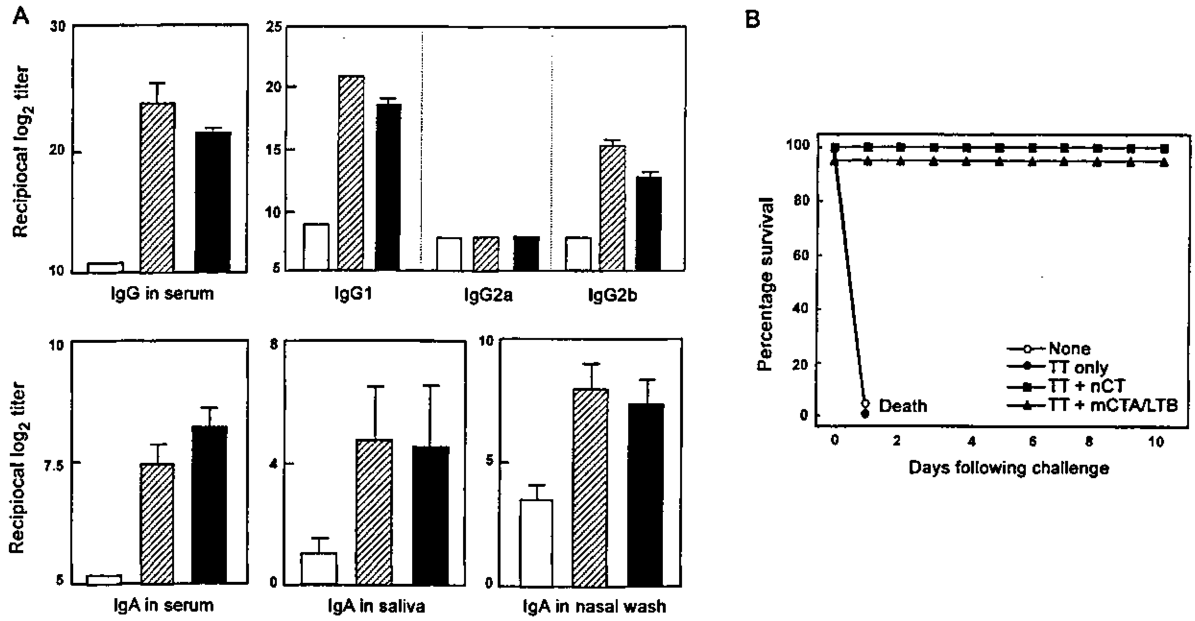

Figure 3. Mucosal adjuvant activity of chimeric adjuvant that combines A subunit of mutant cholera toxin E112K with pentameric B subunit of heat-labile enterotoxin from enterotoxigenic *Escherichia coli* (mCTA/LTB). *A,* Nasally administered mCTA/LTB-supported antigen-specific systemic IgG (including IgG subclass) and mucosal IgA antibody responses. Groups of mice were immunized nasally with 5 μg of tetanus toxoid (TT) alone *(open bars),* with 0.5 μg of native cholera toxin (nCT; *hatched bars*), or with 10 μg of mCTA/LTB *(solid bars)* on days 0, 7, and 14. Samples were collected 1 week after last immunization. Bars represent mean antibody titers ± SE in each group. *B,* Nasal immunization with TT and mCTA/LTB-induced protective immunity against challenge with tetanus toxin. One week after the last immunization, all groups were challenged on day 21 by subcutaneous injection of 130 minimum lethal doses of tetanus toxin in 0.5 mL of PBS including 0.2% gelatin. Each group consisted of 5 mice; data are representative of 2 separate experiments.

fore, we assessed total and TT-specific IgE antibodies at weekly intervals. Both total and TT-specific IgE antibody responses reached maximum levels by day 21 (data not shown). These findings suggest that the levels of IgE induced by the chimera were much lower than those evoked by the native form of CT.

Mucosal protection against influenza virus infection by mCTA/LTB chimera. To determine the ability of mCTA/LTB chimera to support the generation of protective immunity in mucosal compartments, BALB/c mice were immunized nasally with inactivated influenza HA vaccine together with mCTA/LTB chimera and then infected nasally with a lethal dose of PR8 viruses. Interestingly, high levels of anti-PR8 HA IgA antibodies in nasal wash and anti-PR8 HA IgA and IgG antibodies in lung wash were detected after nasal immunization (figure 5*A*). In addition, high levels of anti-PR8 HA IgG antibody responses were detected in serum samples (figure 5*A*). Just as with the antibody responses, virus titers in the lung wash showed that the 2-dose regimen conferred complete protection against infection (figure 5*B*). These results clearly indicate that nasally administered mCTA/LTB is a useful adjuvant against mucosal diseases such as influenza infection.

Discussion

In the present study, we have developed a novel chimera molecule consisting of the A subunit of mCT E112K and the B subunit of LT produced by the *B. choshinensis* host-vector system as a new generation of safe and effective mucosal adjuvants. It is well known that CT and LT are effective adjuvants and that they are capable of enhancing both mucosal IgA and systemic IgG antibody responses to coadministered protein antigen; however, both enterotoxins cause severe diarrhea and thus are unsuitable for use in humans [32–34]. Therefore, several groups, including ours, have developed nontoxic derivatives of CT or LT that may be suitable for use in humans [4–11, 35]. Newly created *B. choshinensis*–derived mCTA/LTB chimera molecule did not induce any increases in intracellular cAMP and failed to elicit fluid accumulation in ligated ileal loops. Furthermore, nasal administration of TT together with mCTA/LTB as adjuvant could elicit TT-specific serum IgG and IgA antibody responses with minimal induction of IgE antibody responses. These mucosally induced serum IgG antibody responses provided complete protection against systemic challenge with tetanus toxin. Even more importantly, influenza vaccine given with this nontoxic chimera adjuvant elicited antigen-specific IgA and IgG antibodies in the lungs and provided complete protection against mucosal infection with influenza virus. These results indicate that this novel chimeric mCTA/LTB molecule is a potent nontoxic mucosal adjuvant for the induction of protective immunity in both mucosal and systemic compartments.

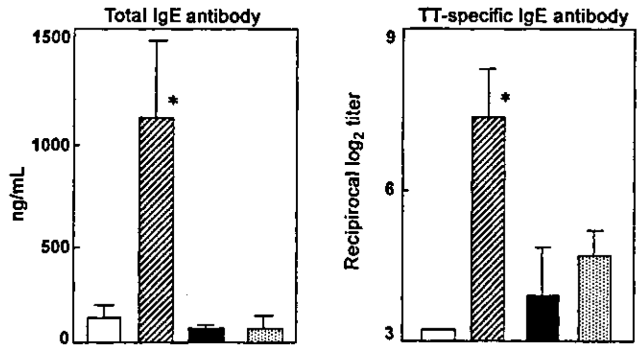

Figure 4. Nasally administered tetanus toxoid (TT) with chimeric mucosal adjuvant that combines A subunit of mutant cholera toxin E112K with pentameric B subunit of heat-labile enterotoxin from enterotoxigenic *Escherichia coli* (mCTA/LTB) did not induce total and TT-specific IgE antibodies in serum samples. Groups of mice were immunized nasally with 5 μg of TT alone *(open bars)*, with 0.5 μg of native cholera toxin (CT; *hatched bars*), with 10 μg of mCTA/LTB *(solid bars)*, or with 10 μg of mCT E112K *(dotted bars)* on days 0, 7, and 14. Serum samples were collected 1 week after last immunization. Bars represent mean antibody titers ± SE in each group. $*P < .05$, compared with that of mice immunized with TT alone.

It has been reported that a number of children are prone to anaphylaxis when given certain live vaccines, such as measles, mumps, rubella, and varicella vaccines [36]. These vaccine preparations include high amounts of gelatin, and the anaphylactic reaction is caused by anti-gelatin IgE antibodies [37, 38]. Therefore, an important criterion for the development of mucosal adjuvants should be to reduce or diminish the potential for IgE antibody production without losing the ability to generate antigen-specific mucosal IgA and serum IgG antibody responses. In a previous study, we demonstrated that among different mutant forms of CT tested, mCT E112K was selected as a safe and effective adjuvant because it supported antigen-specific IgA responses with lower levels of total and anti-CTB IgE antibodies than those observed with different toxin-derived adjuvants after nasal immunization [15]. Interestingly, the present results showed that nasal administration of mCTA/LTB did not enhance IgE antibody responses. Collectively, point mutations in the A subunit of CT may be a key element in the regulation of IgE antibody responses. Current studies are focused on elucidating the molecular basis for the regulation of IgE responses by A subunit that emerged from the present and previous studies. The mCTA/LTB chimera has unique and potentially beneficial features of both enterotoxins and should be considered as a new candidate mucosal adjuvant for future application in humans, which will avoid the danger of anaphylactic shock and/or allergic reactions provoked by IgE antibodies.

Our previous studies have shown that CT accumulates in the olfactory nerves and epithelium regions when given nasally [23]. This uptake of CT has been shown to be ganglioside GM1–dependent. Furthermore, CT as mucosal adjuvant redirects coadministered protein antigen into these neuronal tissues [23]. The finding raised some concerns about a potential role for ganglioside GM1–binding molecules that target neural tissues, including the central nerve system, in nasal immunization. However, a recent study provided new evidence that deposition of CT via the olfactory tissues did not lead to obvious pathologic changes in brain tissue after nasal administration [39]. Although we still do not know the exact biologic and pathologic significance of enterotoxin deposition in the central nervous system mediated by ganglioside GM1–binding of olfactory tissues, we have investigated the distribution of mCTA/LTB and

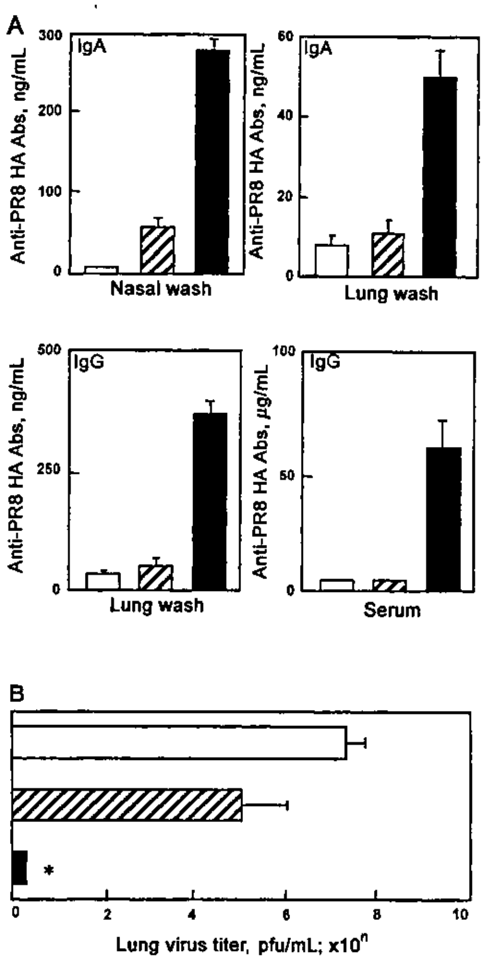

Figure 5. Nasal immunization with inactivated influenza vaccine together with chimeric mucosal adjuvant that combines A subunit of mutant cholera toxin E112K with pentameric B subunit of heat-labile enterotoxin from enterotoxigenic *Escherichia coli* (mCTA/LTB) protected mice from influenza virus infection. BALB/c mice were inoculated nasally without *(open bars)* or with 5 μg of PR8 vaccine together with 5 μg of mCTA/LTB *(hatched bars)*, and second administration was done 4 weeks later *(solid bars)*. Two weeks after second immunization, groups of mice were infected with lethal dose of PR8 influenza virus. Levels of anti-PR8 hemagglutinin (HA) antibodies in nasal wash, lung wash, and serum samples *(A)* and virus titer in lung wash samples *(B)* were assessed 3 days after infection. $*P < .05$, compared with that of nonimmunized mice.

TT in olfactory nerves and epithelium and in olfactory bulbs after nasal immunization. Interestingly, unlike the native form of CT, mCTA/LTB did not influence trafficking of TT into the olfactory nerves and epithelium or into olfactory bulbs, although mCTA/LTB itself accumulated in olfactory central nervous system regions. A separate study has indicated that mCT E112K, the parent molecule of the A subunit in our chimera, also fails to redirect TT into the central nervous system (authors' unpublished data). Thus, our chimeric adjuvant inherits this unique feature of mCT E112K. These results suggest an interesting possibility that redirected trafficking of protein antigen by CT may be mediated by ADP-ribosyltransferase activity. It has been reported that CTB selectively binds to ganglioside GM1, whereas LTB uses several ligands, including ganglioside GM1, ganglioside GM2, and asialo ganglioside GM1, as target cell surface receptors [3]. Thus, an alternative possibility would be that the difference in these binding sites may influence the capacity for redirection antigen trafficking.

In the present study, the *B. choshinensis* host-vector system was chosen for production of chimeric mCTA/LTB molecule, because it has several beneficial properties. First, the *B. choshinensis* expression construct has been shown to be the most effective mass production system for recombinant protein [40]. For example, the quantity of CTB produced by *B. choshinensis* is 1000-fold higher than that by wild-type *V. cholerae* [41]. Second, *B. choshinensis* is a gram-positive bacterium that is known to be nonpathogenic; thus, the possibility of contamination by endotoxin and other virulence factors was excluded. Indeed, our results showed that endotoxin levels of purified mCTA/LTB were at the lower limit of detection (16 EU/mg). Furthermore, mice given mCTA/LTB did not possess any clinical or pathologic symptoms caused by endotoxin (data not shown). These results clearly indicate that the *B. choshinensis* host-vector system has significant advantages in the preparation of mucosal adjuvants, such as the mCTA/LTB chimera, for use in humans.

In summary, a novel chimeric mucosal adjuvant possessing beneficial features of mCTA and LTB was constructed and produced by the *B. choshinensis* host-vector system. Our results have provided evidence that nasal vaccination with TT plus mCTA/LTB elicited antigen-specific mucosal IgA and serum IgG antibodies without IgE responses. Furthermore, TT-specific antibody responses induced by TT plus mCTA/LTB provided protective immunity against challenge with tetanus toxin. It is also important to emphasize that nasal immunization with influenza HA vaccine together with chimeric mCTA/LTB resulted in effective production against viral challenge in the respiratory tract. This newly developed chimeric molecule that combines the A subunit of mCT E112K and the B subunit of nLT should be considered as a novel candidate mucosal adjuvant.

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Autocrine IL-15 Mediates Intestinal Epithelial Cell Death Via the Activation of Neighboring Intraepithelial NK Cells[1]

Naotoshi Kinoshita,* Takachika Hiroi,* Noriyuki Ohta,* Satoshi Fukuyama,* Eun Jeong Park,* and Hiroshi Kiyono[2]*†

Intestinal intraepithelial lymphocytes (IELs), which reside between the basolateral faces of intestinal epithelial cells (IECs), provide a first-line defense against pathogens via their cytotoxic activity. Although IEC-derived IL-7 and IL-15 are key regulatory cytokines for the development and activation of IELs, we report here that IL-15 but not IL-7 mediates the reciprocal interaction between IELs and IECs, an important interaction for the regulation of appropriate mucosal immunohomeostasis. IL-15-treated IELs induced cell death in IECs via the cytotoxic activity in vitro. Among the different subsets of IL-15-treated IELs, $CD4^-CD8^-TCR^-$ IELs, which express NK marker (DX5 or NK1.1), showed the most potent syngenic IEC killing activity. These intraepithelial NK cells expressed Ly-49 molecules, NKG2 receptors, and perforin. These results suggest the possibility that the cell death program of IECs could be regulated by self-produced IL-15 through the activation of intraepithelial NK cells. *The Journal of Immunology,* 2002, 169: 6187–6192.

Intestinal intraepithelial lymphocytes (IELs)[3] reside between the basolateral faces of intestinal epithelial cells. Most IELs are T cells which express either TCR$\alpha\beta$ or TCR$\gamma\delta$ and the majority of IELs express the cytotoxic $CD8^+$ phenotype, either as a CD8$\alpha\alpha$ homodimer or a CD8$\alpha\beta$ heterodimer (1–3). Since the 1980s, several reports have suggested that IELs possess NK-like cytotoxic activity, even without any stimulation (4–6). It turned out that NK-like cytotoxicity partly depends on $CD4^-CD8\alpha\alpha^+$ and $CD4^-CD8^-$ IELs (7). In addition, it has been shown that IELs contain not only T cells but also TCR-negative NK cells in mice, rats, and chickens (7–9). However, our knowledge is limited in regard to the biological significance of TCR^- NK cells in the IEL compartment.

IL-15 is a potent T cell growth factor that uses the IL-2Rβ chain, γ-chain, and its own IL-15Rα chain. IL-15 shares biological activities but no significant sequence homology with IL-2 (10). Unlike IL-2, which is secreted only by T cells, IL-15 mRNA is expressed by non-T cells, including kidney, placenta, skeletal muscle, macrophages, and epithelial cells (11). IL-15 is reported to enhance the proliferation and activation of memory type $CD8^+$ T cells, NK cells, and IELs (12, 13). Analysis of IL-15R$\alpha^{-/-}$ and IL-$15^{-/-}$ mice has demonstrated a critical role for IL-15 in regulating the development and expansion of NK cells and IELs (14, 15).

In this study, we report that IL-15 enhanced the syngenic cytotoxicity of a specific subset of IELs whose phenotype belongs to $CD4^-$, $CD8^-$, TCR^-, and NK marker-positive ($DX5^+$ or $NK1.1^+$) subsets. Moreover, in vitro culture of IELs with IL-15 induced the preferential expansion of these intraepithelial NK cells (NK IELs). These activated NK IELs can induce cell death in intestinal epithelial cell lines via a perforin-dependent pathway. These data suggest the possibility that IL-15 produced by intestinal epithelial cells (IECs) may specifically lead to the activation of NK IELs, which in turn induce the self-killing of IECs via perforin.

Materials and Methods

Mice and cell lines

Six- to 8-wk-old C3H/HeN (H-2^k) and C57BL/6 (H-2^b) mice were purchased from Clea Japan (Tokyo, Japan) and maintained in the animal facility of the Research Institute for Microbial Diseases (Osaka University, Osaka, Japan) for at least 2 wk before the experiments. The murine intestinal epithelial cell line, MODE-K (H-2^k), was a kind gift from Dr. D. Kaiserlian (Institute Pasteur, Lyon, France) (16). CMT-93 (H-2^b) was purchased from Dainippon Pharmaceutical (Osaka, Japan).

Reagents

Human rIL-15 and murine Fas-Fc fusion protein were purchased from R&D Systems (Minneapolis, MN) and murine rIL-7 was purchased from PeproTech EC (London, U.K.). For the flow cytometer analysis and cell sorting, FITC- or biotin-labeled CD4 (RM4-5), FITC- or PE-labeled CD8α (53-6.7), PE- or biotin-labeled CD8β (53-5.8), FITC- or PE-labeled TCRβ (H57-597), FITC- or PE-labeled TCRδ (GL3), FITC-labeled CD3ϵ (145-2C11), purified Fas ligand (FasL; MFL3), biotin-labeled hamster IgG (mixture, G70-204 and G94-56), PE-labeled NK1.1 (PK136), allophycocyanin-labeled streptavidin, FITC-labeled Ly-49AB6 (A1), FITC-labeled Ly-49C and I (5E6), FITC-labeled Ly-49D (4E5), FITC-labeled Ly-49G2 (4D11), FITC-labeled NKG2A/C/E (20d5), and biotin-labeled pan-NK (DX5) Abs were all purchased from BD PharMingen (San Diego, CA), and PE-labeled CD3ϵ Ab was purchased from Serotec (Oxford, U.K.). Concanamycin A (CMA) was purchased from WAKO (Osaka, Japan) as an inhibitor for a perforin-mediated DNA fragmentation assay.

Cell preparations

IELs were isolated by a modified method that has been described elsewhere (17). Briefly, short segments of small intestine were stirred in RPMI 1640 with 2% FBS and 0.5 mM EDTA. After a 15-min incubation, segments were vigorously shaken and mononuclear cells were collected. To obtain lymphocyte-enriched fractions, mononuclear cells were subjected to the Percoll density gradient separation containing 40 and 75% fractions (Pharmacia Fine Chemicals, Uppsala, Sweden). This procedure has been shown to remove intestinal epithelial cells for the enrichment of IELs (18).

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[3] Abbreviations used in this paper: IEL, intraepithelial lymphocyte; IEC, intestinal epithelial cell; CMA, Concanamycin A.

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Culture conditions

IELs were cultured for 3–7 days in complete RPMI 1640 with 10% FBS, 50 μg/ml gentamicin, 100 μg/ml streptomycin, 100 U/ml penicillin, 50 μM 2-ME, 2 mM glutamate, 100 μM nonessential amino acid (Life Technologies, Tokyo, Japan), 25 mM HEPES buffer, and 50 ng/ml human IL-15. In some experiments, 50 ng/ml murine IL-7 instead of IL-15 was added. This dose of IL-7 and IL-15 has been shown to be optimal for the activation of IELs (19). MODE-K was precultured in DMEM (Nacalai Tesque, Kyoto, Japan) supplemented with 50 μg/ml gentamicin, 100 μg/ml streptomycin, 100 U/ml penicillin, and 10% FBS (16).

Isolation of IEL subsets by magnetic cell sorting

IELs cultured for 3 days with IL-15 were separated into three subsets (e.g., $CD4^+$ and $CD4^-CD8\alpha\beta^+$ mixed fraction, $CD4^-CD8\alpha\alpha^+$ fraction, or $CD4^-$ $CD8^-$ fraction) by magnetic cell sorting (auto-MACS; Miltenyi Biotec, Bergisch, Germany). First of all, IELs were harvested from a culture flask and then dead cells were removed using a Dead Cell Removal kit (Miltenyi Biotec). IELs were stained with anti-CD4-biotin and anti-CD8β-biotin for 30 min at 4°C. After being washed, IELs were stained with streptavidin-microbeads for 15 min at 4°C. These cells were then subjected to auto-MACS. The positive fraction contained $CD4^+$ and $CD4^-CD8\alpha\beta^+$ mixed subsets. The negative fraction was further separated into $CD4^-CD8\alpha\alpha^+$ and $CD4^-CD8^-$ lymphocytes by the treatment with CD8α-microbeads (53-6.7: Miltenyi Biotec). Furthermore, $CD4^-CD8^-$ lymphocytes were separated into $CD4^-CD8^-$ TCR^+ fractions and $CD4^-CD8^-$ TCR^- fractions by auto-MACS with biotin-TCRβ and biotin-TCRδ Ab followed by streptavidin-microbeads incubation.

DNA fragmentation assay

A DNA fragmentation assay was performed by using a modified protocol described previously (20). Briefly, MODE-K, which was cultured for 20 h before use in a 96-well culture plate, was pulsed with 10 μCi/well [^{3}H]thymidine for 2 h. Unincorporated [^{3}H]thymidine was removed with PBS containing 2% FBS. MODE-K was incubated with effector cells (e.g., IELs) at various concentrations in flat-bottom 96-well plates in the presence of 50 ng/ml IL-15 or IL-7. After incubation for 6 h, cells were washed with PBS and detached from the culture plate by 0.05% trypsin-EDTA (Life Technologies) to harvest adherent cells. Incorporated [^{3}H]thymidine was quantified using a scintillation counter. [^{3}H]Thymidine-labeled unfragmented DNA was calculated as follows: percent DNA fragmentation = 100 × (1 − cpm experimental group/cpm control group).

Quantitative RT-PCR for measurement of perforin mRNA levels

To measure perforin-specific mRNA levels of freshly isolated and cultured IELs, quantitative RT-PCR was adapted using LightCycler (Roche Diagnostics, Mannheim, Germany) technology as described previously (21). IELs were collected and total RNA was extracted by TRIzol reagent (Life Technologies). To ensure that the same amount of synthesized cDNA was applied, the amount of cDNA labeled with digoxigenin was determined by a chemiluminescent image analyzer (Molecular Imager System; Bio-Rad, Hercules, CA). A detailed protocol for the synthesis of cDNA was previously reported by our laboratory (21). For the amplification of cDNA, 20 μl of PCR mix was added to each tube to give a final concentration of 50 μM 5′ primer, 50 μM 3′ primer, 200 μM FITC-labeled probe, 200 μM LightCycler Red-labeled probe, 4 mM $MgCl_2$, and 1× LightCycler-Fast Start DNA Master Hybridization Probe Mix (Roche Diagnostics). The oligonucleotide primers specific for the perforin (sense, 5′-GACCGCACCT GCACCCTCTGT-3′; antisense, 5′-TGAAGTCAAGGTGGAGTGGAG-3′), perforin detection FITC-labeled probe (5′-CAGGACCAGTAC AACTTT AATAGCGACA-3′), and LightCycler Red 640-labeled hybrid probe (5′-AGTAGAGTGTCGCATGTACAGTTTTCG-3′) were designed and produced by Nihon Gene Research Laboratories (Sendai, Japan). After being heated at 94°C for 10 min, cDNA was amplified for 45 cycles, each cycle consisting of 95°C for 15 s, 62°C for 20 s, and 72°C for 20 s. RT-PCR products of IEL using the primers above were used as an external control. After PCR was completed, LightCycler software converted the raw data into amoles per applied cDNA (1 μg) concentration of target molecules (21, 22).

Results

IL-15 enhanced the cytotoxic activity of IELs against the IEC line

IL-7 and IL-15 produced by IECs can provide a stimulation signal for the proliferation and survival of IELs (23). At first, our investigation was aimed to test whether these cytokines influenced the killing activity of IELs against IECs. Following the incubation of the cytokine-treated IELs isolated from C3H/HeN (H-2^k) mice along with the syngenic IEC line, MODE-K (H-2^k), the level of DNA fragmentation in MODE-K was assessed to determine the extent of cell death. Although freshly isolated IELs showed no killing activity, IL-15-pretreated IELs induced significantly increased levels of DNA fragmentation of MODE-K (Fig. 1). On the other hand, IL-7 pretreatment resulted in only a minimal increase in cytotoxic activity (Fig. 1). These data demonstrate that IL-15 could much more dramatically enhance the in vitro cytotoxic activity of IELs against IECs than could IL-7.

TCR^- IELs showed the most potent killing activity against IECs

Because IELs contain both thymus-dependent and -independent lymphocytes, we next sought to determine which subset of IELs possessed the most potent IL-15-induced killing activity. Following 3 days of culture with IL-15, IELs were separated by magnetic cell sorting into three fractions, including the thymus-dependent $CD4^+$ and $CD4^-CD8\alpha\beta^+$ mixed fraction, thymus-independent $CD4^-CD8\alpha\alpha^+$ fraction, and thymus-independent $CD4^-CD8^-$ fraction. These three fractions were then used as effector cells in the DNA fragmentation assay. Among these three fractions, $CD4^-CD8^-$ IELs showed the most killing function (Fig. 2*A*).

Since the $CD4^-CD8^-$ fraction consisted of TCR^+ (mostly γδT cells) and TCR^- fractions (data not shown) (24), $CD4^-CD8^-$ IELs were further separated into TCR^+ or TCR^- fractions by auto-MACS to investigate which of the two fractions was the more potent killer cell subset. Due to the limited number of cells recovered from the $CD4^-CD8^-$ fraction after auto-MACS cell separation, IELs were initially cultured with IL-15 for >3 days. TCR^- IELs increased up to ~30% of the entire cell population after 7 days of culture (data not shown). DNA fragmentation assays of 7-day pretreated IELs revealed that the $CD4^-CD8^-TCR^-$ fraction is the far more potent killer fraction than the $CD4^-CD8^-TCR^+$ fraction (Fig. 2*B*).

The IL-15-induced IEC killing activity of $CD4^-CD8^-TCR^-$ IELs was also demonstrated by the use of the C57BL/6 strain. CMT-93, derived from rectal carcinoma of C57BL mice, were used as target cells. IL-15-pretreated $CD4^-CD8^-TCR^-$ IELs of C57BL/6 mice showed far more killing activity than any other fractions (Fig. 2*C*).

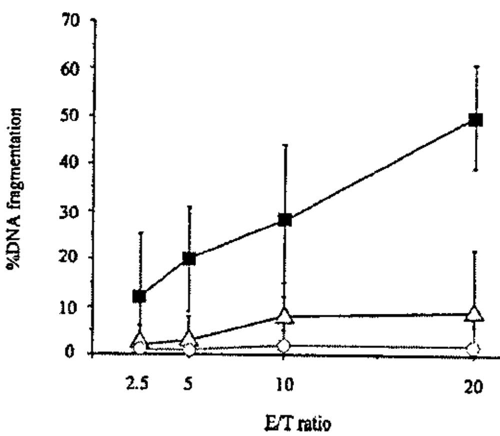

FIGURE 1. IL-15 enhanced the cytotoxic activity of IELs against MODE-K. The IEC line MODE-K was pulsed with [^{3}H]thymidine for 2 h. MODE-K was cultured for 6 h with freshly isolated IELs (○) from C3H/HeN mice and IELs were pretreated for 3 days with 50 ng/ml IL-15 (■) or IL-7 (△) as effector cells in the presence of 50 ng/ml IL-15 or IL-7 at various E:T ratios, respectively. These data are representative of three independent experiments.

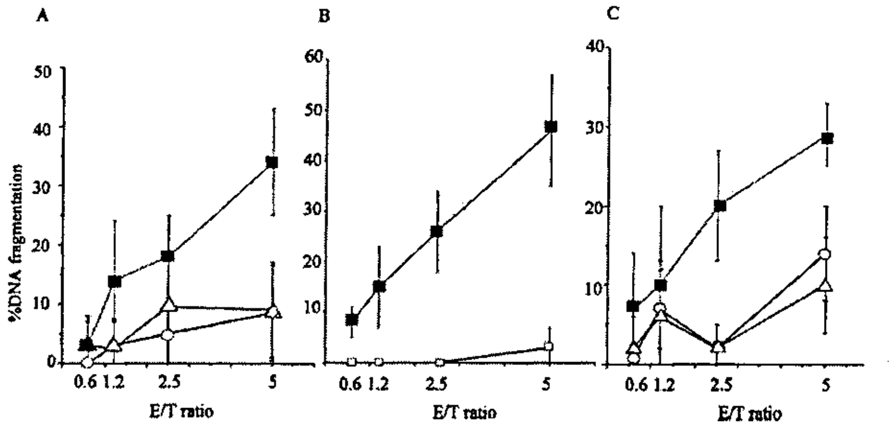

FIGURE 2. CD4⁻CD8⁻TCR⁻ IELs are responsible for the cytotoxic activity against IEC lines. *A*, Whole IELs from C3H/HeN mice cultured for 3 days with 50 ng/ml IL-15 were separated into $CD4^+$ and $CD4^-CD8\alpha\beta^+$ mixed fractions (○), $CD4^-CD8\alpha\alpha^+$ (△), and $CD4^-CD8^-$ (■) fractions by magnetic cell sorting. These three isolated fractions were cocultured with ^{3}H-labeled MODE-K for 6 h in the presence of 50 ng/ml IL-15. *B*, Whole IELs from C3H/HeN mice cultured for 7 days with 50 ng/ml IL-15 were separated into two fractions, $CD4^-CD8^-TCR^+$ (□) and $CD4^-CD8^-TCR^-$ (■). These two separated fractions were cocultured with ^{3}H-labeled MODE-K for 6 h in the presence of 50 ng/ml IL-15. *C*, Whole IELs from C57BL/6 mice were cultured for 7 days with 50 ng/ml IL-15. IELs were then separated into three fractions: CD4 or $CD8\alpha$ positive (○), $CD4^-CD8^-TCR^+$ (△), and $CD4^-CD8^-TCR^-$ (■). These separated fractions were cocultured with ^{3}H-labeled CMT-93 for 6 h in the presence of 50 ng/ml IL-15. These data are representative of three independent experiments.

Inasmuch as the TCR^- fraction showed cytotoxicity against IECs, it was interesting to examine whether this fraction expressed the NK marker. According to flow cytometric analysis, IL-15 but not IL-7 increased the number of TCR^- IELs and these TCR^- IELs are NK marker (DX5) positive (Fig. 3*A*). In the case of C57BL/6 mice, we investigated the expression of NK1.1. As one might expect, the treatment of IELs from C57BL/6 mice with an optimal concentration of IL-15 resulted in the increase of $TCR^-NK1.1^+$ cells (Fig. 3*B*). We next examined the expression of NK receptors of IL-15-treated $TCR^-NK1.1^+$ IELs. When the expression of Ly-49 molecules and NKG2A/C/E was analyzed by flow cytometry, $TCR^-NK1.1^+$ IELs of C57BL/6 mice expressed Ly-49C, D, G2, and NKG2A/C/E but not Ly-49A (Fig. 3*C*). IL-15 treatment enhanced the expression of Ly-49C, D, G2, and NKG2A/C/E but not Ly-49A (Fig. 3*C*). NKG2D mRNA was also detected by RT-PCR both in freshly isolated and IL-15-pretreated TCR^- IEL fractions (data not shown). These results indicate that the TCR^-NK marker $^+$ IEL fraction is responsible for the IL-15-mediated killing of IECs. Inasmuch as mRNA specific for $CD3\epsilon$ and pre-$TCR\alpha$ were not detected by RT-PCR (data not shown) but NK receptors are expressed in this TCR^-NK marker$^+$ IEL fraction (Fig. 3*C*), we referred to this IEL subset as NK IELs.

IL-15 enhanced perforin-mediated cytotoxicity of TCR^-DX5^+ IELs

In general, perforin/granzyme and FasL are major molecules which are involved in the cytotoxicity of NK cells (25). To evaluate the role of perforin in the cytotoxic activity of NK IELs against MODE-K, the level of perforin-specific mRNA expressed by IL-15-treated IELs was examined by real-time quantitative RT-PCR. The IL-15 treatment significantly increased the level of perforin-specific mRNA expression by IELs when compared with the IL-7 treatment (Fig. 4*A*). When perforin expressions by the thymus-dependent $CD4^+$ and $CD4^-CD8\alpha\beta^+$ mixed fraction, thymus-independent $CD4^-CD8\alpha\alpha^+$ fraction, and thymus-independent $CD4^-CD8^-$ fraction were compared after IL-15 treatment for 3 days, the level of perforin mRNA was the highest in the $CD4^-CD8^-$ fraction (data not shown). Furthermore, the level of perforin mRNA was higher in $CD4^-CD8^-TCR^-$ NK IELs than in $CD4^-CD8^-TCR^+$ fractions after 7 days of treatment (Fig. 4*B*). In contrast, it was interesting to note that IL-15 did not induce FasL expression in the NK IEL subset (Fig. 4*C*).

We next performed an inhibitory experiment using the specific antagonist molecules, CMA and Fas-Fc fusion protein, respectively. As an inhibitor of vacuolar type H^+-adenosine triphosphatase, CMA induces the degradation of perforin in T and NK cells (26). IL-15-stimulated IELs were pretreated with 100 nM CMA for 2 h. These CMA-treated IELs were then subjected to the IEC (MODE-K)-DNA fragmentation assay. Pretreatment with CMA inhibited the killing activity of IL-15-stimulated IELs (Fig. 5). We next assessed the role of Fas/FasL signaling for the IL-15-induced IEC killing by IELs. When 20 μg/ml Fas-Fc fusion protein, which has been reported to completely inhibit the Fas/FasL signaling of IELs (20), was added to the culture wells containing MODE-K- and IL-15-pretreated IELs, Fas-Fc fusion protein did not prevent the death of MODE-K induced by IL-15-stimulated IELs (Fig. 5). These findings indicate that IL-15 preferentially up-regulates perforin-mediated cytotoxic activity of NK IELs for the induction of IEC death.

Discussion

A mucosal intranet formed by IECs and IELs provides the first line of defense against pathogens. Furthermore, cell-cell interaction between IECs and IELs is essential for the maintenance of an appropriate immunological homeostasis. IECs have been shown to produce a variety of cytokines and chemokines, including IL-6, IL-7, IL-8, IL-15, stem cell factor, TGF-α, TGF-β, monocyte chemoattractant protein 1, TNF-α, and GM-CSF, which act as communication tools for the mucosal intranet (27–32). Bacterial or viral invasion at the intestinal mucosa results in induction and/or up-regulation of cytokine and chemokine expression (31–33). These biologically active factors are critical for the regulation of both innate and acquired immunity in mucosa. IELs, which consist of thymus-dependent and -independent T cells, have been shown to contain subsets which possess cytotoxicity against bacteria and viruses (3, 34, 35). It should be noted that this cytotoxic activity is provided by both non-MHC-restricted and MHC-mediated Ag-specific manners, corresponding to innate and acquired immunity, respectively (5, 7, 35).

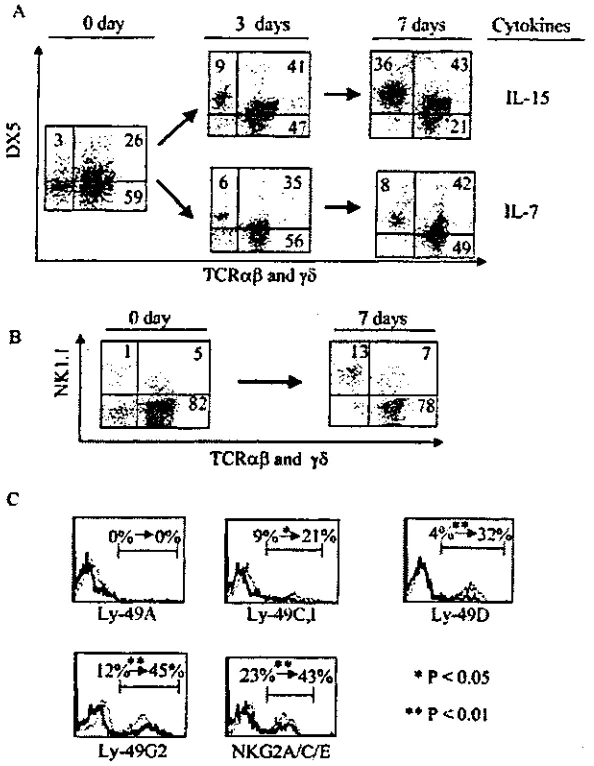

FIGURE 3. Induction of NK marker- and NKR-positive TCR$^-$ IELs by IL-15. *A*, Expression of DX5 on freshly isolated IELs (0 days), on IELs pretreated for 3 days (3 days) and on IELs pretreated for 7 days (7 days) with IL-15 (50 ng/ml) or IL-7 (50 ng/ml) were analyzed by flow cytometer. These data are representative of three independent experiments. *B*, Expression of NK1.1 on freshly isolated and on IELs pretreated with IL-15 (50 ng/ml) for 7 days was analyzed by flow cytometer. *C*, The TCR$^-$NK1.1$^+$ fraction was gated and the expression of Ly-49A, C, D, G2, and NKG2A/C/E were examined. The bold line represents freshly isolated NK IELs and the dotted line represents IL-15-pretreated NK IELs. The numbers show the mean percentage of the positive fraction of freshly isolated and IL-15-pretreated NK IELs. The unpaired *t* test was used for the statistical evaluation.

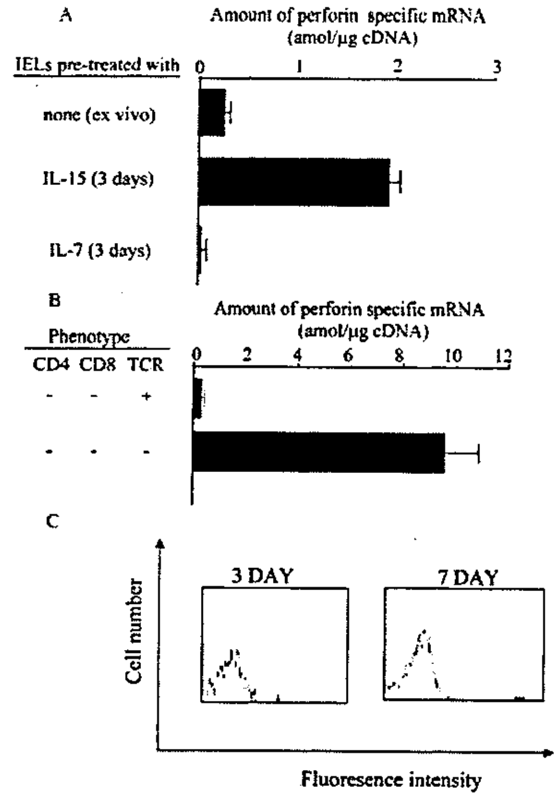

FIGURE 4. IL-15 enhanced the expression of perforin but not FasL in NK IELs. *A*, mRNA was isolated from freshly isolated IELs and IELs pretreated with IL-15 or IL-7 for 3 days. The levels of perforin mRNA were measured by LightCycler. *B*, After a 7-day incubation of whole IELs with IL-15 (50 ng/ml), cells were separated into two fractions, CD4$^-$CD8$^-$TCR$^+$ and CD4$^-$CD8$^-$TCR$^-$, by magnetic cell sorting. mRNA was prepared for the quantitative RT-PCR. These data are representative of three independent experiments. *C*, After IL-15 treatment for 3 or 7 days, the expression of FasL on IELs was analyzed by flow cytometric analysis. The dotted line represents isotype control and the normal line represents FasL. The cells were gated for CD3$^-$ IELs. These data are representative of two independent experiments.

For the appropriate induction and regulation of innate and acquired immunity, the mucosal intranet formed by IECs and IELs utilizes a wide variety of regulatory and inflammatory cytokines and chemokines. In particular, stem cell factor, IL-7, and IL-15 secreted by IECs are key cytokines for the development and stimulation of IELs, especially for thymus-independent IELs (19, 36). In the present study, we have provided new evidence that the cell death program of IECs is preferentially regulated by the self-production of IL-15, which activates perforin-mediated killing provided by CD4$^-$CD8$^-$TCR$^-$ DX5$^+$(or NK1.1$^+$) IELs (NK IELs).

It has been reported that IELs are capable of providing the death signal to IECs in vivo and in vitro. Freshly isolated IELs with high density spontaneously killed freshly isolated syngenic Fas-positive IECs through the FasL-mediated pathway in vitro (37). It was shown that the activation signal provided via the CD3-TCR complex resulted in the augmentation of the FasL expression of IELs. In the case of the mouse graft-vs-host disease model, donor allogenic splenic cells migrated to the IEL fraction and killed recipient IECs through the FasL-mediated pathway (20). TCRαβ$^+$CD4$^-$CD8αβ$^+$ IELs in particular were increased and possessed killing activity through the FasL-dependent pathway in graft-vs-host disease. In addition to the Fas/FasL-mediated cell killing process between IECs and IELs, our present findings demonstrate that the death signal for IECs is also provided via the perforin-dependent system. Our observation that treatment with CMA but not with Fas-Fc fusion protein completely blocked the cytotoxic activity of NK IELs clearly indicates that the killing of IECs by IELs depends on perforin but not on FasL (Fig. 5). Quantitative RT-PCR data further showed that IL-15 enhances the mRNA level of perforin (Fig. 4*B*). The FasL expression of TCR$^-$DX5$^+$ is not up-regulated after cocultivation with IL-15 (Fig. 4*C*). Thus, the IEC death process is regulated via a redundant mechanism of Fas/FasL signaling and a perforin-mediated pathway by two distinct subsets of IELs. TCR$^+$ IELs with high density express FasL for the induction of apoptosis in IECs, whereas TCR$^-$ NK IELs are capable of inducing apoptosis of IECs via perforin following the stimulation signal provided by epithelial cell-derived IL-15. It might be interesting to speculate that these two phases of apoptosis may correspond to acquired and innate immunity, respectively.

Our data suggested that IL-15 can provide both growth- and effector-promoting signals for the TCR$^-$ NK IELs (Figs. 1–3). On the other hand, IL-15 was reported to be a growth- but not effector-promoting cytokine for murine CD8$^+$TCRαβ$^+$ IELs (38). In humans, IL-15 was reported to be the most effective cytokine for the enhancement of cell proliferation, IFN-γ synthesis, and killing by

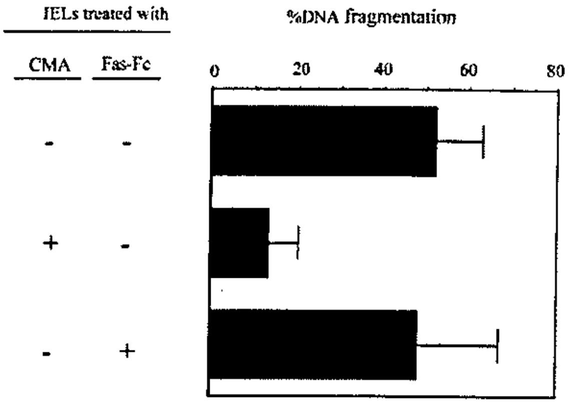

FIGURE 5. The killing activity of IL-15-pretreated IELs depends on perforin. After treatment with IL-15 for 3 days, IELs were preincubated with 100 nM CMA for 2 h. These CMA-treated IELs were cocultured with ^{3}H-labeled MODE-K for 6 h in the presence of IL-15. In addition, IL-15-pretreated IELs were cocultured with ^{3}H-labeled MODE-K for 6 h with 20 μg/ml Fas-Fc fusion protein in the presence of IL-15. These data are representative of three independent experiments.

IELs (23). From these results, we suggest that IL-15 works in a different manner (e.g., growth- and/or effector-promoting effects) among different subsets of effector cells (e.g., NK, $\alpha\beta$T, and $\gamma\delta$T cells) even in the same epithelial compartment.

It was shown that IL-15 enhanced the cytotoxic activity of human IELs and made them more potent killers of the human epithelial cell line (HT-29) (23). The study provided evidence that NK-type IELs are not involved in this cytotoxicity since the numbers of CD16- and CD56-positive NK cells did not change following the stimulation with IL-15. In contrast, our finding provided new evidence that IL-15 can induce cytotoxic activity against IECs in $CD4^{-}CD8^{-}TCR^{-}$ IELs (Fig. 2). These triplet negative lymphocytes possess the most potent killer activity and this fraction expressed an NK marker, DX5 or NK1.1 (Fig. 3). This fraction did not have either CD3ϵ or pre-TCRα mRNA. To further support this finding, our recent and separate study showed that IL-15-treated splenic NK cells ($TCR^{-}DX5^{+}$ cells) deliver a cell death signal to the IEC line (data not shown). T cell-deficient mice, both nude and SCID, have been shown to possess $CD3^{-}$ NK marker-positive IELs (7). These $CD3^{-}NK^{+}$ IELs showed Ab-dependent cell-mediated cytotoxicity as well as cytotoxicity against YAC-1 cells (7). To this end, our recent separate study demonstrated that IL-15-treated NK IELs possess cytotoxic activity against YAC-1 cells (data not shown). According to these data, it is likely that IL-15 is capable of inducing and stimulating NK cells in the IEL compartment for the induction of apoptosis in neighboring IEC.

In general, NK cells do not kill syngenic cells that express the class I MHC molecule (39), probably because inhibitory NKR recognize the class I MHC molecules (40). IL-15 has been reported to participate in the expression of inhibitory NKR, CD94/NKG2A on human $CD8^{+}$ T lymphocytes (41). After stimulation by IL-15, $TCR^{-}NK^{+}$ IELs showed enhanced expression of Ly-49 and NKG2 molecules, which include both activating and inhibitory receptors (Fig. 3*C*). Among murine NK receptors, Ly-49D and NKG2D are activating NKR (42, 43). We observed that anti-Ly49D Ab and NKG2D monomer, which are shown to block the binding of these molecules to their ligands, only partially blocked the death of MODE-K induced by IL-15-pretreated IELs (data not shown). These data may suggest the possibility that unknown activating NKR may be up-regulated by IL-15 and may have a critical role in the cytotoxicity of NK IELs.

NK IELs appear to be heterogeneous in view of the expression of NKR after the treatment with IL-15 (Fig. 3*C*). It has been shown that murine NK cells commonly coexpress at least two or three Ly-49 and NKG2 receptors (44). Furthermore, NK cells expressing one receptor are capable of expressing other NKR, while maintaining expression of the initially expressed receptor (44). Together with our results, it may be possible that IL-15 regulates the expression of different NKR in a nonspecific and cumulative manner.

The production of IL-15 has been shown to be up-regulated after infection with *Salmonella choleraesuis*. (45), *Mycobacterium tuberculosis*, *Toxoplasma gondii* (46), *Listeria monocytogenes* (47), HIV (48, 49), or hepatitis C virus (50). It was reported that after oral infection of rats with *L. monocytogenes*, the level of IL-15 production by IECs was up-regulated and the numbers of $CD3^{-}NK^{+}$ IELs also were increased (47). Our data also demonstrate that IL-15 can expand TCR^{-} NK IELs for the subsequent induction of cell death in IECs. Taken together, these data may also suggest the possibility that up-regulation of IL-15 synthesis enables TCR^{-} NK IELs to kill infected IECs. Removal of infected IECs through the perforin-mediated pathway by IL-15-stimulated NK IELs may represent an important weapon in the mucosal defense system against microbial infection.

In summary, our data demonstrate that 1) IL-15 induces murine intestinal IELs to provide a killing signal to the syngenic IEC line, 2) the NK IEL fraction is the most responsible for the IL-15-induced cytotoxicity, and 3) the IEC killing provided by IL-15-stimulated NK IELs is dependent on perforin. Taken together, these findings suggest an interesting possibility that apoptosis in IECs, at least in part, is induced in a self-regulated way whereby production of IL-15 by IECs itself leads to the activation of perforin-mediated cytotoxicity of NK IELs.

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HIV Mucosal Vaccine: Nasal Immunization with gp160-Encapsulated Hemagglutinating Virus of Japan-Liposome Induces Antigen-Specific CTLs and Neutralizing Antibody Responses[1]

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Nasal immunization of normal mice with HIVgp160-encapsulated hemagglutinating virus of Japan (HVJ)-liposome induced high titers of gp160-specific neutralizing IgG in serum and IgA in nasal wash, saliva, fecal extract, and vaginal wash, along with both Th1- and Th2-type responses. HIVgp160-specific IgG- and IgA-producing cells were also detected in mononuclear cells isolated from spleen, nasal cavity, salivary gland, intestinal lamina propria, and vaginal tissue of nasally immunized mice. In addition, $CD8^+$ CTLs were induced in mice nasally immunized with gp160-HVJ-liposome. These findings suggest that two layers of effective HIV-specific humoral and cellular immunity, in mucosal and systemic sites, were induced by this nasal vaccine. In immunodeficient mice, nasal immunization with gp160-HVJ-liposome induced Ag-specific immune responses for the systemic and mucosal compartments of both Th1 (IFN-$\gamma^{-/-}$) and Th2 (IL-$4^{-/-}$). In vitro Ag-specific serum IgG Ab and vaginal wash samples possessing IgA and IgG Abs that had been induced by nasal immunization with gp160-HVJ-liposome were able to neutralize a clinically isolated strain of HIV-MN strain isolated from Japanese hemophiliac patients. Taken together, these results suggest that, for the prevention and control of AIDS, nasally administered gp160-HVJ-liposome is a powerful immunization tool that induces necessary Ag-specific immune responses at different stages of HIV infection. *The Journal of Immunology,* **2003, 170: 495–502.**

To control HIV infection, it may be possible to develop vaccines that work to block the initial invasion of HIV or to eliminate the virus in patients infected with HIV. Such vaccines may be prophylactic or therapeutic or both. Given that HIV is disseminated mainly during human sexual activity, the mucosal surface of reproductive organs is the primary site of initial invasion and subsequent establishment of the infection in the systemic compartment (1). As a means of resisting HIV transmission through the epithelium of the reproductive organs, to provide a first line of defense against the invasion of HIV, an effective mucosal vaccine is an obvious candidate for development. Mucosal immunization has already been shown to induce Ag-specific immune responses in both the mucosal (e.g., sexual secretions) and systemic (e.g., serum) compartments (2). For inducing Ag-specific immune responses at the sites of sexual contact, nasal administration has been shown to be the most effective route for mucosal immunization (3, 4).

To effectively induce Ag-specific mucosal and systemic immune responses, in this study we used the viral-based hybrid Ag delivery system hemagglutinating virus of Japan (HVJ)[4]-liposome. The HVJ-liposome was developed by combining liposome with fusion proteins derived from the HVJ, also known as the Sendai virus, to enhance the efficiency of Ag delivery to targets (5, 6). In this system, vaccine Ags can be directly introduced into the cytoplasm by virus-cell fusion for the generation of Ag-specific CTLs (7). Moreover, this Ag delivery system has been shown to induce Ag-specific humoral immune responses (8). Thus, for the stimulation of two major arms of immune response including humoral (e.g., Ab) and cell mediated (e.g., CTL), HVJ-liposome is considered to be a useful vaccination Ag delivery vehicle. One interesting possibility entails the inclusion of HIV-surface glycoprotein gp160 into this HVJ-liposome. In a previous study, we demonstrated that, after nasal immunization, HVJ-liposome containing fluorescence-conjugated dextran is effectively taken up by epithelium-associated cells and APCs (8). Thus, HIV-liposome that contains gp160 could be effectively delivered into Ag-sampling M cells, epithelial cells, and APCs in nasopharyngeal-associated lymphoreticular tissue (NALT) and nasal passages (NP). Evidence that nasal immunization with HVJ-liposome containing OVA effectively induces both Ag-specific CTLs and IgA Ab responses (8) suggests that this novel mucosal Ag delivery vehicle could be implemented in the development of an HIV mucosal vaccine.

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[4] Abbreviations used in this paper: HVJ, hemagglutinating virus of Japan; NALT, nasopharyngeal-associated lymphoreticular tissue; NP, nasal passage; SMG, submandibular gland; PP, Peyer's patch; VT, vaginal tissue; SP, spleen; MNp, MN strain isolated from Japanese hemophiliac patients; i-LP, intestinal lamina propria; S-IgA, secretory IgA; CT, cholera toxin.

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In this study, we examined whether nasal immunization with HIV-gp160-encapsulated HVJ-liposome (gp160-HIV-liposome), in conditions in which normal and Th cell functions are deficient, can induce effective HIV-specific immunity in both the mucosal and systemic compartments. To characterize the quality and quantity of HIV Ag-specific immune responses, we tested mucosal gp160-HVJ-liposome with both normal and Th1 (IFN-$\gamma^{-/-}$) and Th2 (IL-$4^{-/-}$) immunodeficient mice. The gp160-HVJ-liposome induced Ag-specific IgG and IgA Abs in both types of mice. Postimmunization samples were also examined in vitro for isotype and subclass distribution as well as neutralizing activity against a clinically isolated strain of HIV. Furthermore, we also examined Ag-specific CTL responses at both mucosal and systemic sites after nasal immunization with gp160-HVJ-liposome. In short, the major aim of the present study was to evaluate the potential for application of the gp160-HVJ-liposome system in the development of a mucosal vaccine to control of HIV infection.

Materials and Methods

Mice

BALB/c mice were obtained from SLC Japan (Hamamatsu, Japan) and BALB/c strain mice with IFN-γ and IL-4 deficiency (IFN-$\gamma^{-/-}$ and IL-$4^{-/-}$) between 6 and 12 wk of age were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in barrier-protected animal facilities under pathogen-free conditions using the ventilated microisolator cage in the experimental animal facility at the Research Institute for Microbial Diseases, Osaka University (Osaka, Japan).

Preparation of HVJ-liposome

The preparation of HVJ-liposome has been described in detail elsewhere (5–8). Briefly, the lipid mixtures (cholesterol, sphingomyelin, dioleoylphosphatidyle-thanolamine, phosphatidylcholine, and phosphatidylserine) were deposited on the side of the flask by removing the chloroform solvent in a rotary evaporator. The dried lipid was hydrated in 200 μl of balanced salt solution (137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl; pH 7.5) containing HIV gp160 (100 μg; Protein Sciences, Meriden, CT). A liposome suspension was prepared by vortexing, sonicating, and shaking to form liposome. Purified HVJ (Z strain) was inactivated immediately before use by UV radiation. The liposome-gp160 complex suspension was incubated with 10,000 hemagglutinating units of HVJ and collected by sucrose density gradient ultracentrifugation. The preparation contained ~1 μg of gp160/10 μl in the wet volume of HVJ-liposome.

Immunization and sampling schedule

Mice (n = 4 per group) were nasally immunized with gp160-HVJ-liposome (1 μg per 10 μl per mouse). Each group of mice was immunized once a week for four consecutive weeks. To monitor the induction of IgG and IgA anti-gp160 specific Abs, serum, saliva, fecal extract, and vaginal wash were collected at day 0 as preimmunization samples and, after the immunization, were collected five times at weekly intervals.

Detection of Ag-specific Ab production by ELISA

HIV-specific Ab titers in serum, saliva, fecal extract, and vaginal wash were determined by ELISA using modified methods as described previously (9). ELISA plates were coated with 100 μl of 2 μg/ml gp160 in 0.1 M carbonate buffer and incubated overnight at 4°C. The plates were then incubated with blocking solution (Block Ace; Dainippon Pharmaceuticals, Osaka, Japan) at 37°C for 2 h. Dilutions of all mucosal secretions starting at 1/4 and serum starting at 1/32 were made with blocking solution, and 50 μl of each dilution was added to duplicate wells of Ag-coated plates. After an incubation at 37°C for 2 h, the coated plates were washed with PBS-polyoxyethylene sorbitan monooleate (Tween 20; Wako Pure Chemical, Tokyo, Japan) and incubated with 100 μl of a 1/4000 PBS dilution of biotin-conjugated goat anti-mouse IgG and IgA detection Ab (Southern Biotechnology Associates, Birmingham, AL) at 37°C for 2 h. The plates were then washed with PBS-Tween 20 and incubated with 100 μl of a 1/2000 PBS-polyoxyethylene sorbitan monooleate (Tween 20; Wako Pure Chemical, Tokyo, Japan) dilution of streptavidin HRP (Life Technologies, Rockville, MD) for 1 h at room temperature. After incubation, color was developed with tetramethylbenzidine (Wako, Tokyo, Japan), stopped with 0.5 N HCl, and measured by OD_{450} on an ELISA reader (Lab System, Helsinki, Finland).

Isolation of mononuclear cells

Mononuclear cells from submandibular glands (SMG), NP, NALT, Peyer's patch (PP), vaginal tissue (VT), and spleen (SP) were isolated as previously described (3, 8–12). In brief, mononuclear cells from NALT and SP were isolated by the mechanical dissociation method using gentle teasing through stainless steel screens. NP, SMG, VT, and PP mononuclear cells were isolated by an enzymatic dissociation procedure using collagenase type IV (Sigma-Aldrich, St. Louis, MO).

Detection of HIV-specific Ab-producing cells by ELISPOT assay

Mononuclear cells were analyzed for Ag-specific Ab production at single cell level using Ag- and isotype-specific ELISPOT assay as previously described (8, 9, 12, 13). Briefly, 96-well filtration plates with a nitrocellulose base (Millititer HA; Millipore, Bedford, MA) were coated with 2 μg of gp160 per well. Single cell suspensions of mononuclear cells from different tissues were added at varying concentrations and then incubated at 37°C for 4 h in air with 5% CO_2 and 95% humidity. After incubation and washing, detection Abs consisting of 1 μg/ml of HRP-labeled goat anti-mouse-μ, -γ, or -α (Southern Biotechnology Associates) were added to the plate. The spots were developed by 3-amino-9-ethylcarbazole (Moss, Pasadena, MD) and counted under a dissecting microscope.

Analysis of HIV-specific T cell responses

For analysis of gp160-specific T cell responses, $CD4^+$ T cells were isolated from NALT, NP, PP, VT, and SP by a magnetic cell sorting system (MACS; Miltenyi Biotec, Auburn, CA) and FACS (BD Biosciences, Mountain View, CA) as previously described (14). Purified $CD4^+$ T cells (>98%) were suspended in complete medium and cultured at a density of 1×10^5 cells/ml in the presence of gp160 (5 μg/ml) and T cell-depleted and irradiated (3000 rad) splenic feeder cells (1×10^6 cells/ml) in flat-bottom 96-well microculture plates (Costar, Cambridge, MA) (15). After 6 days of incubation, using cytokine-specific murine cytokine ELISA kits (Amersham Life Science, Arlington Heights, IL), culture supernatants of Ag-stimulated T cells were examined for the presence of IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-10.

Ag-specific CTL assay

Lymphocytes isolated from nasally vaccinated mice were restimulated in vitro for 5 days with gp160 gene-transfected syngeneic BALB/c3T3 fibroblasts, termed 15-12, expressing an immunodominant epitope identified as a peptide composed of 15 aa (P18IIIB: RIQRGPGRAFVTIGK) (16). A long-term CTL line was also generated by repetitive stimulation of immune cells with mitomycin C-treated gp160 gene-transfected fibroblasts and 10% Con A supernatant condition medium according to a method described previously (17). After culturing for 5 days, the cytolytic activity of the Ag-restimulated cells was measured using a 6-h CTL assay with various ^{51}Cr-labeled targets as previously described (16–18). For testing the peptide specificity of CTLs, effectors and ^{51}Cr-labeled targets were mixed with various concentrations of peptide at the beginning of the assay. Percent-specific ^{51}Cr release was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Maximum release was determined by evaluating supernatants of cells that were lysed by the addition of 5% Triton X-100. Spontaneous release was determined by evaluating target cells incubated without added effector cells. SEMs of triplicate cultures were always within 5% of the mean. To confirm the surface phenotype and class I MHC restriction of the gp160-specific CTLs, the CTL effector cells were variously pretreated with anti-CD4 mAb (rat IgM, RL172.4 hybridoma) plus rabbit complement (Low-Tox Rabbit Complement; Cedarlane Laboratories, Hornby, Ontario, Canada), anti-CD8 mAb (rat IgM, 3.115 hybridoma) plus complement, or complement alone (16). These treated effector cell populations were then tested for killing activity on either fibroblasts transfected with gp160 gene (P18IIIB) or the control fibroblasts (18).

HIV neutralization activity assay

In vitro neutralizing assay of HIV was performed as previously described (19). Briefly, serum IgG Abs were purified from mice immunized with gp160-HVJ-liposome by using protein A-Sepharose (Amersham Biosciences, Little Chalfont, U.K.). Serum IgG was also purified from preimmunized and nonimmunized mice. In addition, vaginal wash was also tested for the presence of neutralizing activity. Appropriately, diluted serum and vaginal wash Abs were incubated with 100 medium tissue culture infective dose units of primary field isolates from Japanese hemophiliac patients (HIV-MNp) (19, 20) for 60 min at 37°C, and the mixture was shaken with 1×10^6 PHA-activated PBMCs for 60 min in a 37°C water

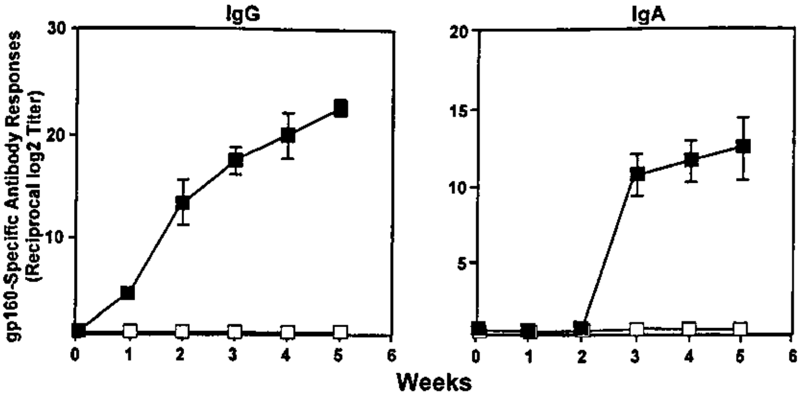


FIGURE 1. Induction of gp160-specific serum IgG and IgA Abs by nasal immunization with gp160-HVJ-liposome. Mice were immunized nasally with gp160-HVJ-liposome (■) (1 μg/mouse of gp160) or HVJ-liposome alone (□) once per week for four consecutive weeks. Serum was collected weekly and analyzed by ELISA for gp160-specific IgG and IgA Abs responses. It should be noted that nasal immunization with gp160 alone did not lead to the induction of Ag-specific IgG response. The levels of gp160-specific Abs are expressed as reciprocal end-point titers. The levels of total serum IgG and IgA Abs were also measured at 5 wk after the initial immunization with gp160-HVJ-liposome (IgG, 850 ± 450 μg/ml; and IgA, 360 ± 130 μg/ml). The levels of total serum IgG and IgA Abs were very similar between mice nasally immunized with gp160-HVJ-liposome and HVJ-liposome alone. Results represent the values (mean ± SEM) for four mice per group from three different experiments.

bath (19). After washing, the cells were cultured in the presence of recombinant human IL-2 (40 U/ml; Shionogi, Osaka, Japan) for 7 days. After the incubation, culture supernatants were subjected to a p24 Ag ELISA (Dynabot, Tokyo, Japan) for the measurement of HIV. Data were expressed as percentage inhibition of p24 Ag production in the culture supernatants compared with results from cultures to which preimmunized or nonimmunized serum IgG or vaginal wash were added. Virus stocks were titrated on PHA-activated normal PBMCs, and the $TCID_{50}$ of each virus was determined as described previously (19).

Results

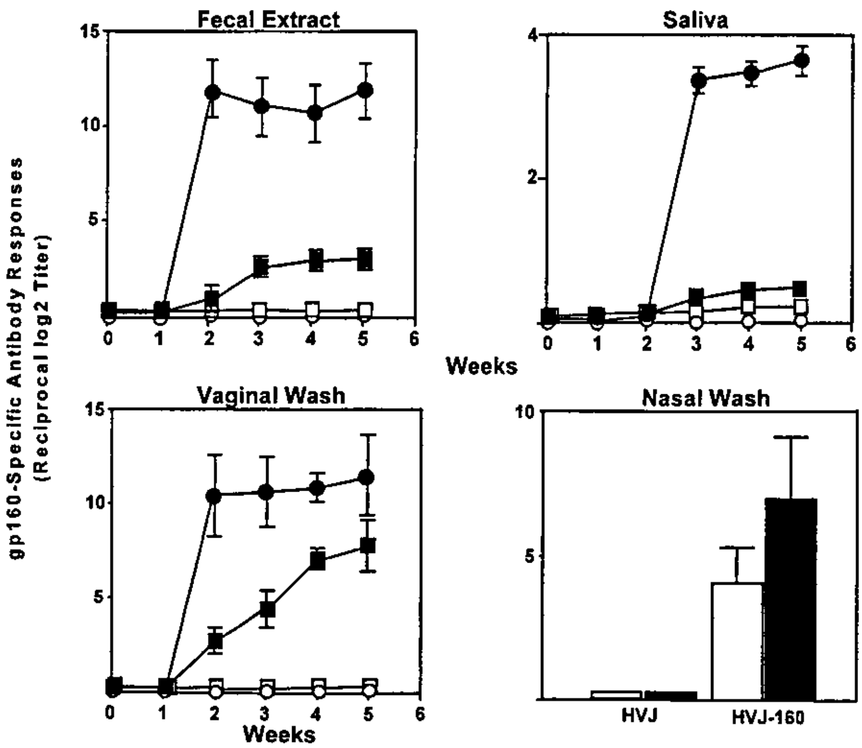
Nasally administered gp160-HVJ-liposome induced Ag-specific Abs in both serum and mucosal secretions

With high titers of gp160-specific serum IgG and IgA Abs already detected between the second and third week after initial immunization, gp160-specific Ab responses were clearly induced by nasal immunization with gp160-HVJ-liposome (Fig. 1). Furthermore, gp160-specific IgA Abs were present in different mucosal secretions including fecal, salivary, vaginal, and nasal (Fig. 2). Relevant to sexual activity, relatively high titers of HIV gp160-specific IgA and IgG Abs were present in the vaginal wash of mice nasally immunized with gp160-HVJ-liposome. Taken together, these results indicate that nasal vaccination with gp160-HVJ-liposome was an effective immunization procedure for the induction of HIV gp160-specific systemic IgG and IgA, as well as mucosal IgA and IgG, immune responses.

Induction of Ag-specific Ab-producing cells by nasal immunization with gp160-HVJ-liposome

The isolation of mononuclear cells from SP and different mucosa-associated tissues (e.g., VT, intestinal lamina propria (i-LP), NP,

FIGURE 2. Induction of gp160-specific mucosal IgA Ab by nasal immunization with gp160-HVJ-liposome. Mice were immunized nasally with 1 μg/mouse gp160-HVJ-liposome (● or ■) or HVJ-liposome alone (○ or □) once a week for four consecutive weeks. Mucosal secretions were collected weekly from reproductive and intestinal tracts as well as oral and nasal cavities and then were analyzed by ELISA for gp160-specific IgG (■ or □) and IgA (● or ○) Ab responses. The levels of gp160-specific Ab are expressed as reciprocal end-point titers. The levels of total mucosal IgA Abs were also measured at 5 wk after the initial immunization with gp160-HVJ-liposome (fecal extracts, 8.5 ± 2.7 μg/ml; saliva, 1.5 ± 0.8 μg/ml; vaginal wash, 7.5 ± 3.9 μg/ml; and nasal wash, 6.2 ± 3.7 μg/ml). Similar levels of total mucosal IgA Abs were noted in mice nasally immunized with HVJ-liposome alone. Results represent the values (mean ± SEM) for four mice per group from three separate experiments.



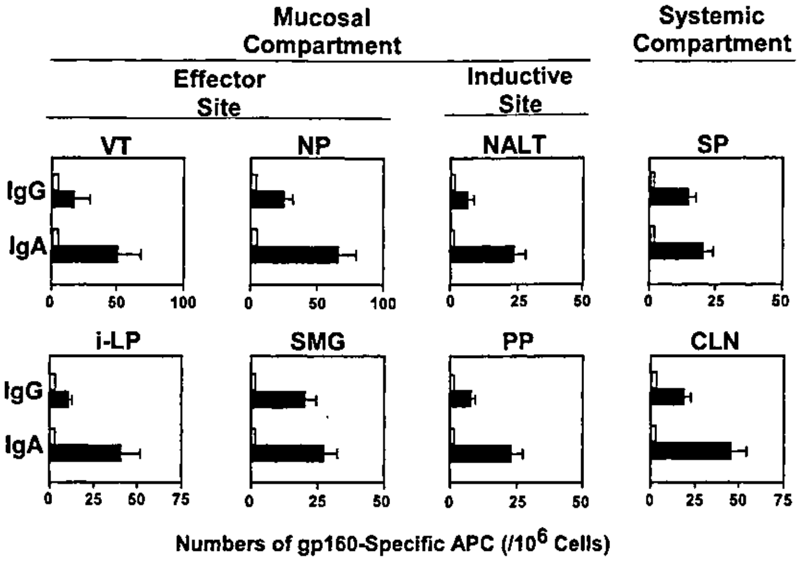


FIGURE 3. Induction of gp160-specific IgG and IgA Ab-producing cells by nasal vaccination with gp160-encapsulated HVJ-liposome. Mice were immunized nasally with gp160-HVJ-liposome (1 μg of gp160/mouse; filled bars) or HVJ-liposome alone (open bars) once a week for four consecutive weeks. After the final immunization, mononuclear cells were isolated from VT, i-LP, NP, SMG, NALT, PP, SP, and cervical lymph node (CLN) for the analysis of gp160-specific IgG and IgA Ab-forming cells by ELISPOT. Results represent the values (mean $\pm$ SEM) for four mice in each experimental group. An identical experiment was repeated on three different occasions.

SMG, NALT, and PP) of mice nasally immunized with gp160-HVJ-liposome provided further corroboration of the induction of Ag-specific Ab responses at the cellular level. Isotype- and Ag-specific ELISPOT assays revealed the presence of increased numbers of gp160-specific IgG and IgA Ab-forming cells in the SP and other parts of the systemic compartment (Fig. 3). In addition, Ag-specific IgG and IgA Ab-producing cells were detected in all of the mucosa-associated tissues, including VT, i-LP, NP, SMG, NALT, and PP (Fig. 3). As anticipated, high numbers of gp160-specific IgA Ab-producing cells were present in mucosal effector tissues, including VT, i-LP, SMG, and NP. These results show that nasal immunization with gp160-HVJ-liposome induced Ag-specific IgG- and IgA-producing cells in both the systemic and mucosal compartments.

In both Th1 and Th2 immunodeficient mice, nasal immunization with gp160-HVJ-liposome induced gp160-specific IgG in serum and vaginal secretions

To examine the potential for the application of nasal gp160-HVJ-liposome in the development of a clinical therapeutic vaccine, Th1 (IFN-$\gamma^{-/-}$) and Th2 (IL-$4^{-/-}$) deficient mice were nasally immunized with gp160-HVJ-liposome, after which increased levels of Ag-specific IgG Ab in serum were induced in these immunodeficient mice (Fig. 4). However, the levels of HIVgp160-specific IgG Abs induced in IFN-$\gamma^{-/-}$ and IL-$4^{-/-}$ mice were lower than in nasally immunized normal mice (Figs. 1 and 4). More specifically, in serum and vaginal wash, although the levels of HIVgp160-specific IgA Abs induced in IL-$4^{-/-}$ mice were lower than in nasally immunized normal and IFN-$\gamma^{-/-}$ mice, IgG Abs levels were similar (Figs. 1 and 4). These results suggest that nasal vaccination with gp160-HVJ-liposome might be an effective way to induce HIV Ag-specific immune responses even when the immunodeficient condition of HIV-infected patients varies during the different stages of progression of AIDS.

Characterization of gp160-specific IgG subclass Abs in mice nasally vaccinated with gp160-HVJ-liposome

We next investigated the IgG subclass of gp160-specific Abs in serum isolated from immunodeficient (IFN-$\gamma^{-/-}$ and IL-$4^{-/-}$) and wild-type mice nasally immunized with gp160-HVJ-liposome. In wild-type mice, gp160-specific IgG2a Ab level was highest, followed by Ab subclasses IgG1, IgG2b, and IgG3 (Fig. 5). As anticipated, Ag-specific IgG1, IgG2b, and IgG3 Abs, but not IgG2a Abs, were induced in IFN-γ-deficient mice (Fig. 5). In samples from IL-$4^{-/-}$ mice, the highest titers of Ag-specific subclass Ab were for IgG2a. Interestingly, high levels of gp160-specific IgG3 Abs were consistently induced in these two immunodeficient types and wild-type mice. The results obtained by the analysis of wild-type mice suggest that the gp160-HVJ-liposome system preferentially induced Th1-type responses based on the profile of the Ag-specific IgG subclass distribution. It is worth emphasizing that the

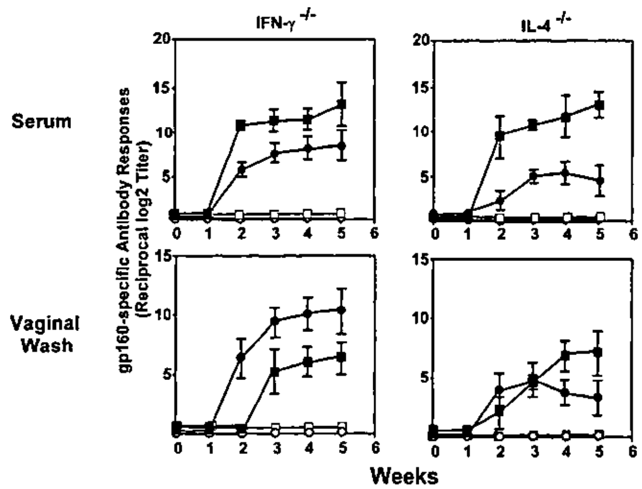


FIGURE 4. Induction of Ag-specific immune responses in Th1- and Th2-deficient mice by nasal vaccination with gp160-HVJ-liposome. IFN-$\gamma^{-/-}$ and IL-$4^{-/-}$ mice were nasally immunized with gp160-HVJ-liposome (● or ■) or HVJ-liposome alone (○ or □) once a week for four consecutive weeks. Serum and vaginal wash were collected weekly and then analyzed by ELISA for gp160-specific IgG (■ or □) and IgA (● or ○) Abs. Responses are expressed as reciprocal end-point titers. The levels of total serum IgG and IgA Abs were examined in IFN-$\gamma^{-/-}$ (IgG, 1010 $\pm$ 450 μg/ml; IgA, 620 $\pm$ 240 μg/ml) and IL-$4^{-/-}$ mice (IgG, 1120 $\pm$ 550 μg/ml; IgA, 260 $\pm$ 120 μg/ml) at 5 wk after the initial immunization with gp160-HVJ-liposome. The levels of total IgA in vaginal wash were also measured in IFN-$\gamma^{-/-}$ (8.8 $\pm$ 3.5 μg/ml) and IL-$4^{-/-}$ (2.9 $\pm$ 1.8 μg/ml) mice nasally immunized with gp160-HVJ-liposome. Results represent the values (mean $\pm$ SEM) for four mice in each experimental group. An identical experiment was repeated on three different occasions.

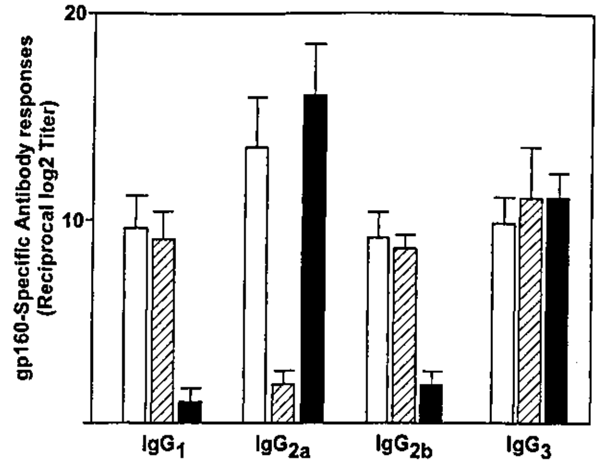


FIGURE 5. Analysis of gp160-specific IgG subclass Ab responses in Th1- and Th2-immunodeficient and wild-type mice nasally vaccinated with gp160-HVJ-lioposome. IFN-$\gamma^{-/-}$ (striped bar), IL-$4^{-/-}$ (filled bar), and wild-type (open bar) mice were nasally immunized with gp160-encapsulated HVJ-liposome once a week for four consecutive weeks. After 1 wk from the final nasal immunization, serum samples were collected and then analyzed by ELISA for gp160-specific IgG subclass Ab responses. Results represent the values (mean ± SEM) for four mice in each experimental group. An identical experiment was repeated on three different occasions.

HVJ-liposome system can overcome Th1- and Th2-deficient conditions and induce selective Ag-specific IgG subclass Ab responses. These results further indicated that nasal vaccination with gp160-HVJ-liposome holds promise for inducing HIV Ag-specific Ab response in immunocompromised patients.

Nasal vaccination with gp160-encapsulated HVJ-liposome induced Ag-specific Th1- and Th2-type responses

To characterize the nature of gp160-specific CD4$^+$ T cell responses, Th1-specific (e.g., IFN-γ and IL-2) and Th2-specific (e.g., IL-4, IL-5, and IL-6) cytokine production was investigated. High levels of Th1-type cytokine (e.g., IFN-γ and IL-2) were detected in the culture supernatant harvested from in vitro gp160-stimulated CD4$^+$ T cells that were isolated from SP and mucosa-associated tissues including VT, NP, NALT, and i-LP from normal mice nasally immunized with gp160-HVJ-liposome (Fig. 6). The findings reflect the results of analysis of Ag-specific IgG subclass responses in wild-type mice nasally immunized with gp160-HVJ-liposome (Fig. 5). A similar Th1 cytokine profile was also apparent in samples from IL-$4^{-/-}$ mice nasally immunized with gp160-HVJ-liposome. With IFN-$\gamma^{-/-}$ mice, as anticipated, the only Th1 cytokine detected was IL-2. It is also worth noting that Th2-associated IgA-enhancing cytokines, such as IL-5 and IL-6, were induced by gp160-specific CD4$^+$ Th cells isolated from systemic and mucosa-associated tissues of all of the mice tested. Taken together, these findings suggested that nasal immunization with gp160-HVJ-liposome induced both Th1- and Th2-type CD4$^+$ T cells in the mucosal and systemic compartments.

Induction of Ag-specific CD8$^+$ CTL responses by nasal immunization with gp160-HVJ-liposome

After confirming that Ag-specific Th1-type responses were elicited by nasal immunization with gp160-HVJ-liposome (Fig. 6), we next tested whether gp160-HVJ-liposome is able to induce MHC class I-restricted gp160-specific CTL responses. At first, CTL activity against gp160 was examined in splenic lymphocyte samples isolated from mice nasally immunized with gp160-HVJ-liposome.

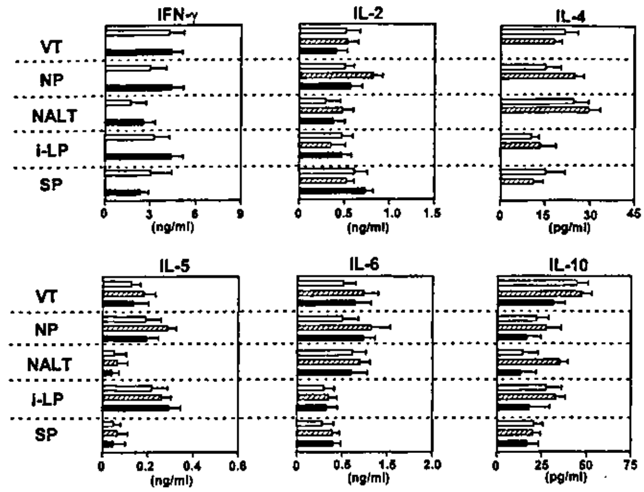


FIGURE 6. Induction of Ag-specific Th1- and Th2-type responses in IFN-$\gamma^{-/-}$, IL-$4^{-/-}$, and wild-type mice by nasal immunization with gp160-HVJ-liposome. CD4$^+$ T cells were isolated from NALT, NP, PP, VT, and SP in wild-type (open bars), IFN-$\gamma^{-/-}$ (striped bars), or IL-$4^{-/-}$ (filled bars) mice nasally immunized with gp160-HVJ-liposome. Purified CD4$^+$ T cells were suspended in complete medium in the presence or absence of gp160 (50 μg/ml), then cultured at a density of 1×10^6 cells/ml together with T cell-depleted and irradiated splenic feeder cells (1×10^5 cells/ml) in flat-bottom 96-well microculture plates. After 6 days of incubation, culture supernatants from individual wells were assayed by cytokine-specific ELISA for the production of IFN-γ, IL-2, IL-4, IL-5, IL-6, and IL-10.

After in vitro restimulation of the immune splenic lymphocytes with gp160 gene-transfected 15-12 fibroblast stimulator cells, we found that the presence of gp160-specific CTL response was detected in mice nasally immunized with gp160-HVJ-liposome (Fig.

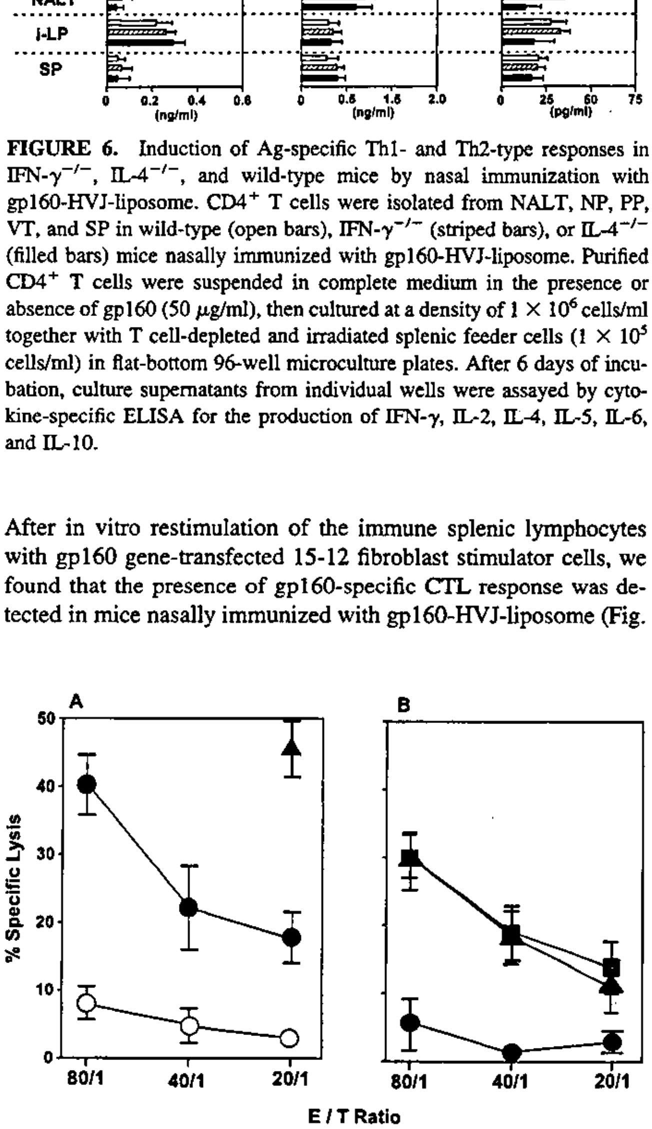


FIGURE 7. Induction of gp160-specific CTL activity by nasal immunization with gp160-HVJ-liposome. *A*, Lymphocytes were isolated from the SP of normal mice immunized with gp160-HVJ-liposome and restimulated with mitomycin C-treated 15-12 fibroblasts for 5 days to enhance the frequency of Ag-specific CTLs (wild-type mice; ●). As a negative control, lymphocytes isolated from the SP of wild type (○) immunized only with HVJ-liposome were used. As a positive control, line IIIB cells were used (wild type; ▲). CTL activity was measured by ^{51}Cr-releasing assay (*A*). *B*, To confirm the surface phenotype and MHC class I restriction of the gp160-specific CTL, we performed specific subset deletion CTL assays. Complement treatment along with the relevant mAbs was conducted to disrupt CD8 T cells (●) and CD4 T cells (■). As a control, splenic lymphocytes were treated with complement only (▲). These selected T cell subsets were then subjected to the CTL assay.

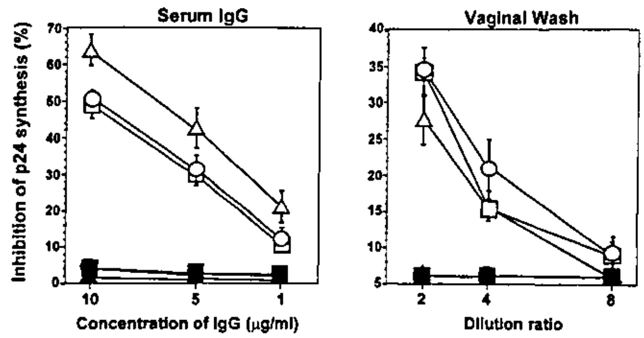


FIGURE 8. Induction of gp160-specific neutralizing Ab responses by gp160-HVJ-lioposome nasal vaccine. In vitro neutralization of HIV-MN was performed by a standard p24 release assay. Six weeks after the start of the nasal immunization regimen with gp160-HVJ-liposome, serum IgG samples and mucosal IgA samples from vaginal washes were prepared from wild-type mice (△) and immune-deficient mice (IFN-$\gamma^{-/-}$, ○; IL-$4^{-/-}$, □). These samples were analyzed for the presence of neutralizing Ab after testing against a clinically isolated strain (HIV-MNp). As a control, serum and mucosal samples from preimmunized mice and mice nasally immunized only with HVJ-liposome were obtained and tested (wild-type mice, ▲; IFN-$\gamma^{-/-}$, ●; IL-$4^{-/-}$, ■). These control samples did not detect any Ag-specific neutralization activity. Results represent the values (mean ± SEM) of four mice in each experimental group.

7). When these splenic lymphocytes were pretreated with mAb anti-CD4 or anti-CD8, the removal of gp160-specific CTL activity was subsequently achieved by anti-CD8 mAb treatment (Fig. 7). These findings suggest that nasal immunization with gp160-HVJ-liposome resulted in the priming of Ag-specific $CD8^+$ CTL responses.

Neutralization of HIV-1 by gp160-specific Abs from mice nasally immunized with gp160-HVJ-liposome

To better characterize virus neutralization activity, a well-characterized, clinically isolated strain of HIV-MNp that expresses IHIGPGRAFY at the core sequence of the HIV principal neutralizing determinant (19, 21) was used as the vial source. Serum IgG and vaginal secretory IgA (S-IgA) Ab samples obtained from wild-type mice nasally immunized with gp160-HVJ-liposome possessed neutralizing activity against HIV-MNp (Fig. 8). Similar neutralization activity was observed with IgG immune serum purified from both IFN-γ- and IL-4-deficient mice that had been nasally vaccinated with gp160-HVJ-liposome (Fig. 8). Preimmunized IgG and vaginal wash samples containing S-IgA, however, showed no neutralizing activity (data not shown). These results indicated that nasal immunization with gp160-HVJ-liposome was capable of strongly inducing gp160-specific IgG and S-IgA Abs with virus-neutralizing activity.

Discussion

An effective and safe prophylactic HIV vaccine that targets both systemic and mucosal immunity could play a decisive role in combating the spread of HIV infection through the epithelium of reproductive organs. Research has already shown that vaginal secretions containing locally produced S-IgA and serum-derived IgG Abs contribute to the formation of first line of defense at the epithelium of reproductive organs (22, 23). For example, Ag-specific S-IgA and IgG in vaginal wash samples have been shown to possess neutralizing activity against viruses or bacteria associated with sexual transmission (3, 24). One of the significant findings of the present study is the demonstration of the induction of HIV-specific IgA and IgG Ab responses in both the systemic and mucosal compartments at sites of sexual contact. We also found that these Ag-specific Abs that were induced by nasal immunization with HVJ-liposome containing HIV gp160 possessed neutralizing activity against a clinically isolated strain of HIV-MNp. In generating these effective mucosal IgA and systemic IgG Abs, nasally administered gp160-HVJ-liposome induced appropriately balanced Th1- and Th2-type $CD4^+$ T cell responses. In addition, HVJ-liposome that contained HIV gp160 elicited Ag-specific CTL responses. These findings indicated that HVJ-liposome is an effective nasal Ag delivery system for the induction of both humoral (e.g., S-IgA and serum IgG) and cell-mediated (e.g., CTL) immunity in the mucosal and systemic compartments.

Nasal administration has been shown to be as effective at inducing Ag-specific immune responses, including Ab production and CTL activity, in both the mucosal and systemic compartments as oral immunization (15, 22, 23, 25, 26). Our own previous studies and work by other researchers has shown that coadministration of mucosal adjuvant is essential for the generation of Ag-specific mucosal and systemic immune responses via the respiratory and gastrointestinal immune system (3, 9, 13, 15). Thus, our previous report has shown that nasal vaccination with the fimbrial protein of *Porphyromonas gingivalis* and a well-known mucosal adjuvant cholera toxin (CT) induces protective immunity via the generation of Th2 cell-mediated Ag-specific mucosal IgA Ab responses (15). Nasal vaccine containing fimbrial protein alone, however, did not lead to the induction of Ag-specific Th and B cell responses (15). Furthermore, when female rhesus macaques were nasally immunized with p55 of SIV in the presence or absence of CT as a mucosal adjuvant (3), nasal vaccine containing both p55 and CT induced Ag-specific IgA and IgG Abs in mucosal secretions (e.g., cervicovaginal, rectal, and salivary), but similar Ag-specific immune responses were not detected in primates nasally immunized solely with p55 (3).

In contrast with the previous investigations discussed above, HVJ-liposome effectively induces Ag-specific Th1 and Th2 cells together with the associated IgG and IgA Ab responses in both mucosal and systemic sites without coadministration of mucosal adjuvant (e.g., CT or *Escherichia coli* heat-labile enterotoxin). It is pertinent to consider why HVJ-liposome induces mucosal and systemic immune responses without mucosal adjuvant. One reason may be its ability to directly deliver the Ag to cells associated with the mucosal immune system. It was shown that HVJ-liposome directly attaches to the cell membrane using envelope glycoproteins, the so-called fusion proteins of the Sendai virus (27–29), which function during natural infection via the respiratory epithelium (30, 31). Furthermore, in a previous study we found that HVJ-liposome is able to preferentially attach to nasal epithelia and deliver encapsulated Ag directly to the surface of epithelial cells, M cells, and APCs (8). The immunoenhancing activity of envelope glycoproteins composed with F and H proteins may bypass the need for adjuvant. For example, it has been shown that virus-associated fusion proteins possess immunoaugmentation or adjuvant activity (8, 29). Other reports have shown that the liposome inherently possesses adjuvant activity (32, 33). Moreover, there is evidence that, after Sendai virus infection of an epithelial cell line (NIH 3T3), overexpression of NF-κB gene is induced, which leads to the up-regulation of IFN-β mRNA induction (34). Therefore, it is also possible that HIV-liposome is involved in the activation of cytokine synthesis that enhances immune response. Taken together, this evidence of the multiple immunoenhancing activities of HIV-liposome is encouraging for the development of a novel and effective Ag delivery vehicle that generates HIV Ag-specific mucosal and systemic immune responses without administration of any additional mucosal adjuvant.

HVJ-liposome, a novel viral-based hybrid consisting of liposome with Sendai virus fusion proteins, has proved effective as a delivery vehicle for introducing DNA into a host (5–7). Not only is HVJ-liposome capable of delivering the encapsulated oligodeoxynucleotide DNA into cell cytoplasm (5–8), this delivery also results in presentation of MHC class I-mediated peptide that induces cell-mediated immune responses (29). Nasal immunization with HVJ-liposome also induces Ag-specific CTL responses at systemic lymphoid tissues in an MHC class I-dependent manner (8). Direct intracellular delivery of Ag via the fusion process demonstrated an encapsulated Ag to the MHC class I pathway (8, 29). In addition to these MHC class I-mediated CTL responses, nasal immunization with gp160-HVJ-liposome induced high levels of gp160-specific $CD4^+$ Th1 and Th2 cell-mediated Ab responses in both systemic and mucosal tissues. This result suggests that mucosally administered HIV-liposome can activate MHC class II-mediated $CD4^+$ T cell responses for the subsequent induction of gp160-specific IgG and IgA Abs. To this end, our previous study demonstrated that HVJ-liposome is capable of presenting peptide Ag together with MHC class II molecules of epithelial cells and macrophages (8). Other evidence from previous studies has also shown that mucosal epithelial cells are able to present Ags (35, 36). Thus, nasally administered HIV-liposome appears to use the mucosa-associated Ag presentation pathway for the maximum induction of gp160-specific IgA immune response. These different findings, taken together, provide evidence that gp160-HVJ-liposome effectively enhances gp160-specific MHC class I-mediated $CD8^+$ CTL and class II-regulated $CD4^+$ Th1/Th2 cell activities.

The established cytokine profile of Ag-specific Th1 and Th2 cells predicts the presence of IFN-γ- and IL-4-dependent IgG subclass Ab responses in mice nasally immunized with gp160-HIV-liposome, and our findings show a profile of Th1 and Th2 cytokines in line with anticipated IgG subclass Ab responses, which indicates that nasally administered HVJ-liposome causes simultaneous induction of Ag-specific serum IgG1 and IgG2a Ab responses. It was long ago shown that IFN-γ is the key cytokine for generation of IgG2a Abs and that IL-4 is the key cytokine for IgG1 Abs (37). Our current results corroborate our previous finding that nasal immunization with OVA-fusogenic liposomes results in the generation of both the Th1 and Th2 cytokine-mediated IgG2a and IgG1 responses (8). Even so, our previous and current investigations of the induction of Ag-specific IgG3 subclass Ab responses differ. Whereas in the previous study Ag-specific IgG3 Abs were not induced in wild-type mice nasally immunized with HIV-liposome containing OVA (8), in the current study, using an identical Ag delivery vehicle, HIV Ag-specific IgG3 Ab responses were in evidence. We do not have any conclusive explanation, other than that the discrepancy may be due to the differing nature of the antigenicity of OVA and gp160.

One of the most significant findings of this study is that nasal immunization with gp160-HVJ-liposome is capable of inducing high levels of gp160-specific serum IgG and mixed S-IgA and IgG in vaginal wash samples isolated from both in immunodeficient Th1 type (IFN-γ) or Th2 type (IL-4) mice and in wild-type mice (Fig. 4). In human AIDS, it has been debated whether Th1- and Th2-type $CD4^+$ T cells are predominantly depleted in HIV-infected individuals. It has been reported that, during the development of HIV infection, there is a change from Th1 to Th2 responses (38). However, another study suggested that a switch from Th1 to Th2 responses does not occur during the progression of HIV infection (39). Meanwhile, it has been shown that HIV preferentially replicates within $CD4^+$ T cells with phenotypical Th0 and Th2 cytokine synthesis (40). Although the exact state of $CD4^+$ T cell deficiency is unknown, we are making provisions for this in the development of a mucosal vaccine that can offer induction of effective Ag-specific immune responses in HIV-infected patients at different stages of $CD4^+$ T cell deficiency. At the same time, we are concerned with developing a therapeutic vaccine system that is able to effectively counteract different Th-type cell deficiency profiles in AIDS. Because nasal administration of the gp160-HVJ-liposome vaccine results in the induction of Ag-specific mucosal IgA and IgG as well as serum IgG Abs in both Th1- and Th2-type immunodeficient mice, our current findings show promising prospects for the application of gp160-HVJ-liposome in the development of a therapeutic mucosal vaccine (Figs. 4 and 6).

This study has also shown that nasal vaccination with gp160-HVJ-liposome is capable of inducing high levels of Ag-specific neutralizing Abs in both serum and mucosal secretions in both wild-type mice and Th1- and Th2-type immunodeficient mice (Fig. 8). The rapid clearance of HIV from the peripheral blood after primary HIV infection is generally thought to be the result of the development of neutralizing Abs or the formation of Ag-Ab complexes (41). The majority of neutralizing Abs detected in HIV-infected individuals react with epitopes that are present on HIV gp120 and gp41 (42). These Abs have been shown to neutralize either a single particular isolate (type specific) or several isolates with varying degrees of sequence homology (group specific). One recent report has in fact demonstrated in vitro that the vaginal wash obtained from mice vaginally immunized with inactivated HIV-1 captured Con A-immobilized polystyrene nanospheres contained an in vitro neutralizing activity against the strain used for the immunization (43). Furthermore, other reports suggest that nasal immunization of mice with gp160 protein is capable of inducing systemic and mucosal HIV-1-neutralizing Abs (25, 44, 45). These results have shown that, after mucosal immunization with Ags and adjuvant, vaginal wash secretions are capable of neutralizing HIV. However, in fact, mucosal Ag-specific Abs were not induced by systemic immunization with different forms of HIV Ags, including gp160 and gp41 (44, 45). The current study found that nasal immunization with gp160-HVJ-liposome in the absence of coadministered adjuvant induces, in vaginal secretions, neutralizing Abs against a clinically isolated strain of HIV-MNp. Because vaginal wash samples contained both S-IgA and IgG Abs, it would be interesting to know which isotype of Ag-specific Abs preferentially provides protective immunity at the reproductive epithelium. To directly address this issue, it is important to purify individual isotypes of Ag-specific Abs from vaginal washes. We were unable to do this in the current study, because the amount of sample was limited. A future task remains: the elucidation of whether a particular isotype or both have proneutralizing activity in the reproductive compartment of mice nasally immunized with gp160-HIV-liposome.

In summary, for the prevention and control of HIV infection, our present investigation has demonstrated the potential prophylactic and therapeutic usefulness of a nasal vaccine based on gp160-HVJ-liposome. It is especially significant that, as well as systemic CTL activity, gp160-HVJ-liposome can induce high levels of gp160-specific mucosal IgA or IgG and systemic IgG Abs with neutralizing activity.

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CD40L in autoimmunity and mucosally induced tolerance

Commentary

See related article, pages 261–267.

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The administration of soluble antigens to the oral or nasal mucosa can lead to systemic unresponsiveness to a subsequent challenge with the same antigens (1). Such mucosally induced tolerance probably protects the body from hypersensitivity reactions to food proteins, pollen, and commensal bacterial antigens present in the normal mucosa and thus helps maintain an appropriate immunological balance between the host and its normal flora. Numerous molecular and cellular inhibitory mechanisms are involved in this unique and important immunological phenomenon. Several lines of evidence show that mucosally induced tolerance does not occur passively, by means of anergy, clonal deletion, or the simple absence of autoreactive immune cells, but also involves ignorance, receptor downregulation, or active cellular suppression (ref. 2; Figure 1a).

The fact that mucosally induced tolerance can suppress antigen-specific T cell proliferation, delayed-type hypersensitivity responses, and antibody production suggests attractive approaches for the prevention and control of autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, encephalomyelitis, and type 1 diabetes (3). However, although mucosal administration of different autoantigens has been tested in human trials, this strategy has not yet yielded a successful therapy (4, 5). Some previous studies have suggested that this failure of human trials was due to concomitant induction of harmful cytotoxic T lymphocytes (CTLs) by mucosally administered antigen (6, 7).

In an attempt to overcome this obstacle, as reported in this issue of the *JCI*, Hänninen et al. (8) have induced a temporary blockade of the interaction between the costimulatory protein CD40 and its ligand CD40L. This interaction has been suggested to foster mucosally induced tolerance by several mechanisms, as shown in Figure 1a. In their present report, the authors show that blocking antibodies to CD40L can prevent the development of antigen-specific CTL responses without affecting the development of oral tolerance.

Hänninen and colleagues (8) tested this approach in rat insulin promotor-OVA (RIP-OVA) mice, a diabetic transgenic strain in which the expression of the model antigen chicken ovalbumin (OVA) in the pancreatic β-islet cells provokes an autoimmune response to these cells. The consequent destruction of these islet cells thus provides a model for the events seen in type I diabetes. Oral administration of a high dose of OVA can promote mucosally induced tolerance, but this treatment also elicits CTL specific for OVA. However, Hänninen et al. observed that transient blockade of CD40L in this system prevented CTL responses without affecting mucosally induced tolerance toward OVA, either in wild-type or in RIP-OVA transgenic mice (Figure 1b). Furthermore, the severity of diabetes, as measured, for example, by blood glucose, was significantly improved after transient blockade of CD40L, suggesting that modulation of CD40L-CD40 interaction could be used clinically to uncouple the desired systemic, autoantigen-specific unresponsiveness from the oral induction of CTLs.

Costimulation in mucosally induced tolerance

These striking data thus appear to indicate that the CD40L-CD40 interaction is dispensable for mucosally induced tolerance. We suggest, however, that it would be premature to rule out a contribution of CD40L and other costimulatory factors to mucosally induced tolerance. In an earlier study, we found that systemic unresponsiveness to either of two model antigens (OVA or hen lysozyme) occurred in *CD40L*$^{+/+}$ wild-type controls but could not be induced in *CD40L*$^{-/-}$ mice (9). Significantly, although these *CD40L*$^{-/-}$ mice have multiple functional defects, such as a failure of germinal center formation, Ig class switching, and the inability to elicit Th1-type responses (10, 11), their antigen-specific T cell responses are intact, as can be seen following priming with myelin basic protein in CFA (12). For this reason *CD40L*$^{-/-}$ mice provide a useful animal model for clarifying the role of CD40L-CD40 interaction in mucosally induced tolerance.

The effect of CD40L in this response may be partially explained by the involvement of another costimulatory pathway, involving the antigen-presenting cell-borne (APC-borne) B7 proteins (also known as CD80 and CD86) and T cell-borne counter-receptors CTLA-4 and CD28. Thus, Samoilova et al. (13) report that blockade of the B7-CTLA-4 interaction can completely abrogate mucosally induced tolerance, whereas blockade of both CD28 and CTLA-4 interactions with B7 molecules has a similar but less dramatic effect. Hence, a proper costimulatory signal generated by CTLA-4 is required for mucosally induced tolerance (Figure 1a), whereas CD28 can apparently activate T cell responses to mucosally derived antigens. In addition, other studies show that stimulation of CD40 with CD40L activates expression of the B7 proteins CD80 and CD86 on the APC surface, thus favoring the interaction of these cells with CD28- or CTLA-4-bearing CD4 T cells (14).

Consistent with this model, Perez et al. (15) observed that the interaction of the B7 proteins with CTLA-4 provides negative signals that limit T cell activation.

Hence, our finding (9) that the lack of CD40L-CD40 interaction blocks the induction of tolerance could reflect the loss of negative signals provided by CTLA-4–CD80/CD86 interaction. The absence of such an effect following treatment with anti-CD40L mAb, as reported by Hänninen et al. (8), may be explained if the doses used were insufficient to block this negative signal cascade completely (Figure 1b). Alternatively, the discrepant findings may reflect the different developmental histories of the animals studied. In particular, the deletion of the *CD40L* gene led to the disorganization and dysfunction of Peyer's patches, organs that are central to the induction of the mucosal immune system (10) and represent an important site for the initiation of oral tolerance (16). The transient antibody blockade used by Hänninen et al. (8), conversely, would not be expected to duplicate this effect on Peyer's patch development.

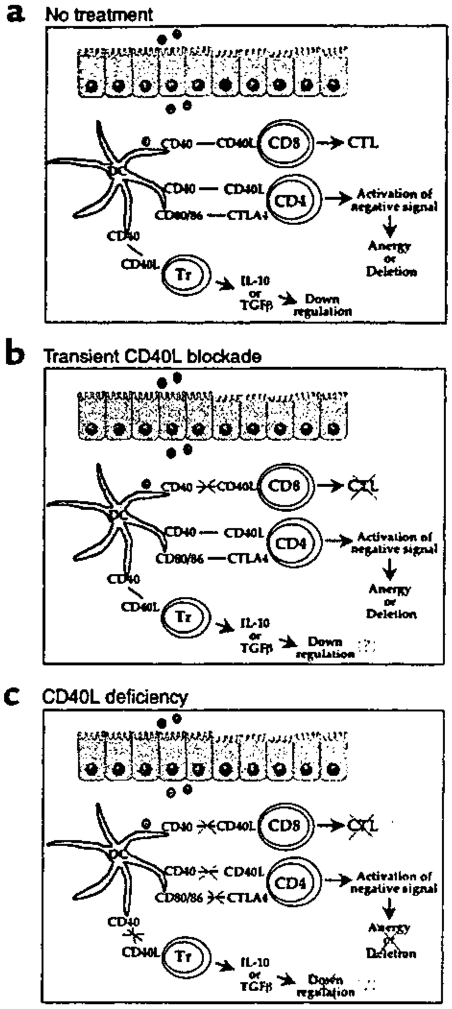

Figure 1

Models for the effects on mucosally induced tolerance of perturbing the CD40L-CD40 interaction. (**a**) In the untreated wild-type mouse, CD40 on dendritic cells (DCs) and other APCs interacts with CD40L expressed on T cells of several subsets, including CD4 and CD8 cells, as well as a CD25 CD4 population of regulatory T cells (Tr). This interaction can lead to both active, antigen-specific CTL responses and the suppression of Th function and inflammation. The latter effects appear to be implicated in mucosally induced tolerance to dietary antigens and endogenous bacteria. (**b**) As shown by Hänninen et al. in this issue of the *JCI* (8), transient treatment with mAb anti-CD40L can prevent induction of CTLs, while apparently leaving some of the other responses intact. Thus, mucosally induced tolerance is not compromised by this treatment. (**c**) In case of the permanent blockage of CD40L-CD40, as occurs in *CD40L*$^{-/-}$ mice, the additional loss of some immunosuppressive signaling cascade and/or of a population of Tr cells prevents mucosally induced tolerance.

CD40L in T cell development

Perhaps as a result of its proposed effects on the expression of costimulatory molecules, CD40 signaling appears to alter the course of T cell development. Kumanogoh et al. (17) recently reported that an important regulatory T cell subtype, the CD25$^+$CD4$^+$CD45RB^{low} T cells (shown as Tr in Figure 1), which can downregulate the pathogenic CD4$^+$CD45RB^{high} T cell subset, is markedly reduced in *CD40*$^{-/-}$ mice. These authors also found that T cell autoreactivity is significantly increased in these animals. Interestingly, adoptive transfer of T cells isolated from *CD40*$^{-/-}$ mice can trigger autoimmune diseases in the recipient *nu/nu* mice in association with enhanced levels of various autoantibodies. Further, APCs isolated from the *CD40*$^{-/-}$ mice fail to induce regulatory T cells producing high levels of the suppressive cytokine IL-10. Another group recently showed that a similar population of regulatory T cells can be induced after challenge with oral antigen (18). After alloantigen stimulation, these cells express CD40L at high levels, raising the possibility that CD40L-CD40 interaction helps regulate their alloantigen-specific immune responses (19). Hence, in the absence of CD40L-CD40 interactions (9), depletion of the population of these regulatory T cells may reduce the expression of IL-10 and TGF-β, immunosuppressive cytokines that promote mucosally induced tolerance (Figure 1c). In the case of transient blockade of CD40L-CD40 signaling (8), conversely, the inhibitory signal provided by regulatory CD25$^+$CD4$^+$ T cells may remain intact (Figure 1b).

CD40L and CTL responses to mucosal antigens

Several recent studies show that, in addition to its tolerogenic effect, oral or nasal administration of soluble autoantigens can generate antigen-specific CTLs (6, 20). The current report from Hänninen et al. (8) confirms these data and suggests possible mechanisms for the effect on CTL function. CD40L- and CD40-mediated interactions between CD4 T cells and mucosal dendritic cells (DCs) might contribute to this potentially