Dendritic Cells Recruitment and In Vivo Priming of CD8+ CTL Induced by a Single Topical or Transepithelial Immunization Via the Buccal Mucosa with Measles Virus Nucleoprotein

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The buccal mucosa, a prototype of pluristratified mucosal epithelia, contains a network of directly accessible class II+ epithelial dendritic cells (DC), similar to skin Langerhans cells. We showed that a single buccal immunization with measles virus nucleoprotein (NP), by either topical application onto or intradermal injection in the buccal mucosa, induced in vivo priming of protective class I-restricted specific CD8+ CTL. Both routes of immunization with NP induced a rapid recruitment of DC into the mucosa, which peaked at 2 h and decreased by 24 h. Treatment of mice with Flt3 ligand resulted in an increased number of DC in the buccal mucosa and enhanced the frequency of IFN-γ-producing NP-specific effectors and the NP-specific CTL response generated after buccal immunization with NP. Finally, NP-pulsed bone marrow-derived DC induced NP-specific IFN-γ-producing cells upon adoptive transfer to naive mice. These data demonstrate that a viral protein delivered to DC of the buccal mucosa induces in vivo priming of protective anti-viral CD8+ CTL. The Journal of Immunology, 2001, 167: 384–391.

Dendritic cells (DC) are considered as the first line sentinels in immune surveillance of peripheral tissues, including epithelia of the skin and mucosal surfaces. The efficiency of DC to initiate an immune response against infectious disease is due to their constant trafficking between peripheral tissues and draining lymph nodes. DC residing in mucocutaneous epithelia are specialized in the uptake and processing of foreign Ag. Following migration to draining lymph nodes, DC acquire expression of costimulatory molecules and become efficient at presenting antigenic peptides loaded onto MHC class I and class II molecules to naïve CD8+ and CD4+ T cells, respectively (1). Adoptive transfer experiments have documented the capacity of DC to induce priming of class I-restricted CTL responses to endogenous allogeneic or infectious Ags in vivo (2) as well as cross-priming of CD8+ CTL when pulsed with exogenous nonreplicating Ags, including inactivated virus (3), hepatitis B surface Ag particles (4), apoptotic cells (5), and exosomes (6).

An important issue for the development of an anti-infectious mucosal vaccine is whether DC within mucosal epithelia are involved in priming CD8+ effector T cells or may favor the development of mucosal and systemic tolerance. Indeed, although DC are present in both Peyer’s patches (7) and lamina propria of the small intestine (8), the efficiency of oral immunization with protein Ags to prime CD8+ CTL responses is hampered by Ag dilution/degradation in the gastrointestinal tract as well as by oral tolerance. Thus, evidence that the delivery of a protein Ag to DC at epithelial surfaces is efficient at inducing CD8+ CTL cross-priming in vivo has been poorly documented.

The buccal mucosa offers several advantages for in vivo analysis of the stimulatory function of epithelial DC after capture of protein Ags. These include permeability of the pluristratified epithelium devoided of a horny layer, direct accessibility of Langerhans cells (LC) populating the epithelium, and induction of delayed-type hypersensitivity responses. LC represent the major APC present in the buccal mucosal epithelium, as shown in humans by the presence of Birbeck granule and the expression of CD1a and HLA-DR molecules (9). LC of the human (10) and rat (11) buccal mucosa can process and present Ags in vitro and seem to be even more efficient than freshly isolated epidermal LC to stimulate allogeneic T cells (12). In the mouse, buccal epithelial LC express, similarly to epidermal LC, a high density of MHC class II molecules and low levels of CD11c and DEC-205 (13).

Transepithelial delivery of replicating as well as nonreplicating Ags through the buccal mucosa has proven to be efficient at inducing specific CTL. Indeed, a single topical buccal immunization with a recombinant vaccinia virus (VV) encoding measles virus (MV) hemagglutinin (HA) or transepithelial buccal injection of a recombinant naked DNA plasmid encoding MV-HA, induced HA-specific CTL (14). Likewise, buccal immunization with inactivated MV could prime mice for a specific CD8+ CTL response, which...
was enhanced in transgenic mice expressing the human MV receptor, CD46 (D. Kaiserlian, unpublished observations). Moreover, contact sensitivity (CS) can be induced by topical application of haptenants on the buccal mucosa and is mediated by hapten-specific CD8\(^+\) CTL independently of CD4\(^+\) T cell help, similarly to CS induced by skin sensitization (13, 15). Buccal LC were involved in hapten capture and transport to draining cervicomandibular lymph nodes (16). Moreover, DC isolated from cervicomandibular lymph nodes of buccally sensitized mice could transfer hapten-specific CS to naive mice (16, 17).

We thus tested whether DC of the buccal mucosa could present a viral protein, either topically applied onto or injected through the buccal mucosa, and induce priming-specific CTL and protection against virus challenge. We found that a primary CTL response could be generated in vivo by mucosal immunization with low doses of MV nucleoprotein (NP) and that DC contributed to in vivo priming of specific CD8\(^+\) T cells.

Materials and Methods

Mice

BALB/C female mice (6–8 wk old) were purchased from Ifa Credo (L'Arbresle, France)

MV recombinant NP

Recombinant MV NP was obtained by infection of insect cells with a baculovirus NP recombinant as previously described (18). The total protein concentration of recombinant NP was determined by the protein titration kit (Bio-Rad, Richmond, CA). Endotoxin levels were <10 pg/ml recombinant NP as assessed by the Limulus amebocyte lysate assay. The L.\(^2\)-restricted NP peptide 281–289 was synthesized by NeoSystem (Strasbourg, France).

Immunizations

Mice were heavily anesthetized by ketamine/xylazine, laid on their sides, and immunized through the buccal mucosa by either transepithelial injection or topical application onto the buccal epithelium (inner faces of cheeks) of 15 µl/cheek of either 1 mg/ml NP (30 µg/mouse, unless otherwise stated) diluted in PBS or PBS alone, as described previously (13). This procedure avoided unexpected swallowing or inhalation of Ag. Cutaneous immunizations were performed by administrating 30 µg NP in 100 µl PBS, either injected s.c. in the dorsal skin or topically applied to the shaved dorsal skin.

Immunohistochemical analysis

Crystat sections (5 µm) of the buccal mucosa were stained with rat anti-class II mAb (CD311) (19) or rat IgG as control. Sections were incubated for 1 h at room temperature with CD311 (1/10 dilution of supernatant) or 10 µg/ml rat IgG as a control. The slides were washed in PBS/1% BSA. Specific binding was revealed by 30-min incubation with mouse absorbed F(ab')\(_2\) goat anti-rat IgG conjugated to biotin (Dako, Carpenteria, CA), followed by 30-min incubation in streptavidin-biotin peroxidase (Dako), according to the manufacturer’s instruction. The reaction was developed using 3-amino-9-ethylcarbazole substrate (Sigma) in citrate buffer, pH 5. The results are expressed as the number of MHC class II\(^+\) cells per square millimeter of tissue.

Anti-CD4 Ab treatment and Flt3 ligand (FL) treatment

Mice were depleted of CD4\(^+\) T cells by i.p. injections of 50 µg anti-CD4 mAb (GK1.5) (20) on days −3, −1, 2, and 7 with respect to day 0 of immunization. Control mice were injected with an irrelevant rat IgG mAb. FACS analysis using PE-conjugated CD4 mAb (Caltag, Tebu, France) showed that CD4\(^+\) T cell depletion was >99% in both peripheral blood and secondary lymphoid organs. Mice were treated with 10 µg FL (provided by Immunex, Seattle, WA), injected i.p. for 9 consecutive days; control mice received PBS alone.

NP-specific CTL assay

NP-specific CTL activity was determined on day 10 after NP immunization after in vitro restimulation of spleen cells with NP-transfected P815 (21) as previously described (14). Briefly, 10\(^6\) spleen cells from immunized BALB/C mice were cultured at 37°C in 24-well culture plates (Falcon; BD Biosciences, Meylan, France) with 10\(^5\) mitomycin C (25 µg/ml)-treated P815-NP cells in a total volume of 2 ml complete DMEM medium. Five days later, half the supernatant was replaced by fresh medium, and the cytotoxic activity was tested on day 7 of culture.

P815 and P815-NP target cells were radiolabeled for 90 min at 37°C with 50 µCi Na\(^{25}\)CrO\(_4\) (sp. act., 1 Ci/mM)/10\(^6\) cells and washed three times in DMEM containing 1% FCS. In some experiments, NP-transfected Ltk\(^−\) cells and EL-4 (H-2\(^b\)) cells pulsed with NP peptide 281–289 were used as negative controls. Target cells (5 × 10\(^5\) cells/well) were cocultivated in 96-well round-bottom plates, with various numbers of spleen effector cells to give E:T cell ratios of 100:1, 30:1, 10:1, 4:1, or 1:1. Anti-L-123 (mAb 30.5.7S; Cedarlane Laboratories, Hornby, Ontario, Canada), anti-KD (mAb 34.1.2S), anti-D (mAb 34.4.20S) (American Type Culture Collection, Manassas, VA), or anti-CD8 (H35) mAbs were added to some wells during the assay to block CD8\(^+\) T cell-mediated cytotoxicity. After 4-h incubation at 37°C, the radioactivity released in the supernatant was determined using a gamma counter (1470 Wizard; Wallac, Gaithersburg, MD). The percentage of cell lysis was calculated as follows: ([experimental cpm – spontaneous cpm]/total cpm – spontaneous cpm) × 100. Spontaneous and total counts per minute were determined from target cells incubated with medium alone or with 100 µl 1 M HCl, respectively.

NP-specific IgG ELISPOT assay

Leukocyte suspensions from spleen and cervicomandibular lymph nodes were depleted in RBC by hypotonic shock in 0.83% ammonium chloride; pulmonary leukocytes were isolated after lung perfusion of the right ventricles with chilled HBSS and digestion of tissue fragments with collagenase/dispase as previously described (22). The resulting suspension contained <3% epithelial cell contamination. The number of NP-specific IgG-producing cells was determined by an ELISPOT assay on day 10 after immunization as previously described (22). Briefly, recombinant NP (10 µg/well) in sodium carbonate buffer, pH 9.6, was coated on nitrocellulose-bottomed wells of 96-well plates (Millipore, Bedford, MA) by overnight incubation at 4°C. The wells were washed three times with PBS and saturated with RPMI 1640 medium containing 10% FCS. Four-fold serial dilutions of 100 µl spleen cell suspension were added to each well and incubated overnight at 37°C. The plates were then washed three times with PBS containing 0.05% Tween 20 (PBS-T) and incubated overnight with biotin-conjugated goat anti-mouse IgG or IgA Ab (Sigma, La Verpillière, France) diluted in PBS-T. Unbound Abs were removed by washing with PBS-T. One hundred microliters of peroxidase-conjugated streptavidin diluted in PBS-T was added to the wells and incubated for 2 h at room temperature. After further washing with PBS-T, the spots were developed using 3-aminio-9-ethylcarbazole substrate (Sigma) in citrate buffer, pH 5. The results are expressed as the number of spot-forming cells (SFC) per 10\(^6\) cells.

NP-specific IgG1 and IgG2a ELISA

Serum IgG1 and IgG2a Abs specific for NP were titrated by ELISA using NP-transfected Ltk\(^−\) cells (21) as previously described (23). Briefly serum dilutions were incubated for 90 min at 37°C with acetone-fixed NP-transfected Ltk\(^−\) cells. Binding of the specific Abs was revealed with biotinylated anti-mouse IgG1 and IgG2a using the streptavidin-alkaline phosphatase system (Sigma). Results are expressed with reference to control anti-NP mAbs used to standardize the assays for IgG1 and IgG2a. Titers were calculated with the SOFTmax program (Molecular Devices, Menlo Park, CA), and the results are expressed as nanograms per milliliter of specific Ab.

Intracerebral challenge

Mice were challenged by intracerebral inoculation with 4 × 10\(^7\) PFU/mouse of a neuroadapted strain of canine distemper virus (CDV) (24). The mice were observed for 1 mo after the challenge for clinical signs of disease, including fur roughing, weight loss, and neurological signs. Death only occurred in the initial acute phase of the disease (i.e., days 9–17 after challenge). The results are expressed as percent survival.

Immunization with bone marrow-derived DC (BM-DC) pulsed with NP

BM-DC were generated from bone marrow progenitors as previously described (25). Briefly, bone marrow was flushed from tibia and femur and depleted of RBC with 0.83% ammonium chloride. The cells were extensively washed in HBSS and cultured at 37°C in 24-well culture plates (10\(^6\) cells/ml/well) in complete RPMI 1640 medium supplemented with 50}
ng/ml recombinant murine GM-CSF (PeproTech, Tebu, Le Peray-en Yvelines, France). Half the medium was replaced every other day by fresh medium plus GM-CSF. Day 7 BM-DC (consisting of 70% CD11c<sup>+</sup>CD11b<sup>−</sup> and 30% CD11c<sup>−</sup>CD11b<sup>+</sup>) were pulsed with 100 μg NP/ml for 3 h at 37°C; the cells were then washed in HBSS, and 10<sup>5</sup> cells were transferred by s.c. injection into naive syngeneic recipient mice.

**IFN-γ ELISPOT assay**

Spleen cells (20 × 10<sup>6</sup>) harvested from either naive or NP-immunized mice were restimulated overnight with 2 × 10<sup>6</sup> P815-NP or P815 cells. The number of NP-specific IFN-γ-producing cells was determined by ELISPOT assay as previously described (26). Briefly, graded numbers of cells were incubated for 4 h at 37°C in duplicate wells of nitrocellulose 96-well plates (MAHA N4510; Millipore) coated with the anti-IFN-γ mAb R46A2. The plates were washed three times with PBS, 0.1% Tween 20 before addition of biotinylated anti-IFN-γ Ab, AN18. The hybridomas producing mAbs R46A2 and AN18 were provided by DNAX (Palo Alto, CA). IFN-γ SFC were developed using streptavidin-alkaline phosphatase (Roche, Gipf-Oberfrick, Switzerland), incubated for 2 h, and extensively washed before addition of 5-bromo-4-chloro-3-indolyl-phosphate (Sigma), as substrate. The number of IFN-γ SFC was counted in each well using a binocular, and the results are expressed as the number of IFN-γ SFC per 10<sup>6</sup> cells.

**Results**

**Priming of CD8<sup>+</sup> CTL by topical or transepithelial immunization with NP through the buccal mucosa**

A single buccal immunization with NP (30 μg), delivered by either topical application or transepithelial injection, was able to induce an NP-specific CTL response (Fig. 1a). Dose-response analysis showed that CTL priming could be obtained with doses of NP as low as 100 ng/mouse, although topical immunization required a higher dose of NP than transepithelial injection for inducing optimal CTL activity (Fig. 1b). The NP-specific CTL response generated by buccal immunization was mediated by MHC class I-restricted CD8<sup>+</sup> effector T cells. This was revealed by the lack of CTL activity against targets of irrelevant haplotypes transfected with NP (Ltk-NP) or with EL4 cells pulsed with the L<sup>d</sup> peptide NP<sub>281–289</sub> (Fig. 1c) and by the complete inhibition of CTL activity in the presence of anti-CD8 mAb or anti-L<sup>d</sup>, but not anti-K<sup>d</sup>-D<sup>d</sup>- or D<sup>d</sup>-specific mAbs (Fig. 1d). These data demonstrated that buccal immunization with NP can prime specific class I-restricted CD8<sup>+</sup> CTL.

Analysis of NP-specific SFC on day 10 after buccal immunization showed the presence of NP-specific IgG-producing B cells only in draining cervicomandibular lymph nodes, not in spleen or lung (Fig. 2A). This was associated with the presence of NP-specific IgG1 and IgG2a in the serum, with higher levels of IgG2a compared with IgG1 (Fig. 2B). These data supported that buccal immunization resulted from NP transport from the buccal via afferent lymph to draining cervicomandibular lymph nodes for local priming of both B and T cells.

**Comparison of NP-specific CTL induction by buccal mucosa and cutaneous immunization**

The efficacy of NP-specific CTL priming by the buccal route was compared with that induced by cutaneous immunization. Subcutaneous injection of NP induced a CTL response comparable to that obtained by buccal transdermal injection (Fig. 3). Alternatively, epicutaneous immunization with NP was ineffective at generating a CTL response. No CTL response was induced by intragastric immunization with NP, demonstrating that NP-specific CTL priming induced by buccal immunization did not merely result from Ag penetration in the digestive tract. These data demonstrated that the buccal mucosa represents a unique site of immunization where, in contrast to skin, a single topical application of protein can induce CTL priming.

**Cross priming of NP-specific CTL by buccal immunization does not require CD4<sup>+</sup>T cell help**

To examine whether induction of NP-specific CTL by buccal immunization required CD4<sup>+</sup> T cell help, BALB/c mice were treated with a depleting anti-CD4 mAb before and after immunization with NP, as described in Materials and Methods. Fig. 4 shows that the NP-specific CTL response developed in CD4-depleted mice, although at E:T cell ratios below 10:1, 5–10 times more effector cells were required to reach optimal levels of cytotoxicity. Thus, CD4 help does not seem to be mandatory for priming NP-specific CTL after buccal immunization with NP.

**Buccal immunization with recombinant NP protects from lethal CDV challenge**

Previous studies have demonstrated that NP-specific CTL induced by parenteral immunization with recombinant VV encoding a class I epitope of MV-NP can protect mice against lethal challenge with a neuroadapted strain of CDV, a Morbillivirus whose NP shares the same dominant L<sup>d</sup> epitope (27). We took advantage of this model to examine the protective role of NP-specific CTL generated by buccal immunization with recombinant NP. Mice immunized by either injection or application of 30 μg NP administered
by the buccal route were challenged 2 mo later by intracranial injection of CDV. Control unimmunized mice injected with PBS alone died between days 10 and 17 after CDV challenge. In contrast, all mice immunized by injection of NP through the buccal mucosa were protected up to 12 mo after CDV challenge. Approximately 50% of the protected mice developed mild clinical signs of disease, including weight loss and fur roughing, whereas the other 50% of the survivors remained free of clinical symptoms throughout the study. Buccal immunization performed by application of NP to the buccal mucosa resulted in 40% protection against challenge and progressive weight recovery (Fig. 5). These data demonstrated that the NP-specific CTL response induced by buccal immunization could protect mice against lethal CDV challenge.

FIGURE 3. Comparison of the NP-specific CTL response induced by buccal and cutaneous immunization routes. Mice were immunized with 30 μg NP administered via buccal mucosa, cutaneous, or intragastric routes as indicated, and the NP-specific CTL response in spleen was determined on day 10 after immunization after in vitro restimulation with P815-NP as described in Fig. 2. The results are expressed as the percent NP-specific cytotoxicity at the E:T cell ratio of 100:1 and are representative of two experiments. *, p < 0.05 (as determined by Student’s t test).

FIGURE 4. NP-specific CTL response induction in CD4+ T cell-depleted mice. Mice treated on days −3, −1, 0, 2, and 7 with anti-CD4 mAb (GK1.5) or control rat IgG were immunized on day 0 by transepithelial injection of 30 μg NP into the buccal mucosa. On day 10 after immunization, the NP-specific CTL response in spleen was determined after in vitro restimulation of effector cells with P815-NP transfectant, and NP-specific cytotoxicity was determined as described in Fig. 1. The results are representative of two experiments using six mice per group.
immunization with NP is sufficient to protect mice from the lethal effect of virus challenge.

**DC are recruited into the buccal mucosa epithelium upon local NP immunization**

Immunohistochemical staining of cryostat sections of the buccal mucosa with anti-class II mAb showed that in normal mice DC are localized in the suprabasal layers of the stratified epithelium as well as in the dermis and the underlying connective tissue (Fig. 6a). Transepithelial injection of NP through the buccal mucosa induced within 30 min the recruitment of strongly class II+ cells with a typical DC-like morphology in suprabasal epithelium and dermis. The maximum increase in the number of DC was observed at 2 h (Fig. 6, b and d) and returned to initial levels by 24 h (Fig. 6, c and d). Similar recruitment was observed after topical application of NP to the buccal mucosa (data not shown). Alternatively, endotoxin-free OVA (15 μg/cheek) did not affect the number of buccal DC. The possibility that the low level of endotoxin in NP inoculum (<0.15 pg) contributed to NP-induced recruitment of DC was ruled out, as buccal administration of LPS at doses up to 10^6 times higher (i.e., 0.1 μg) did not affect the number of buccal DC (data not shown).

Semi quantitative analysis (Fig. 6d) revealed a roughly 2-fold increase in the number of MHC class II+ cells in buccal mucosa (epithelium and dermis). CD11b+ cells accounted for ~50% of the cells accumulating in the dermis at 2 h (data not shown).

**FL treatment causes DC expansion in the buccal mucosa and enhances the NP-specific CTL response**

FL is an hemopoietic factor that promotes in vivo differentiation of DC from hemopoietic progenitors (28). To determine whether increasing the number of mucosal DC could enhance in vivo priming of NP-specific CD8+ T cells, mice were treated for 9 consecutive days with 10 μg FL and were then immunized through the buccal mucosa with NP. Immunohistochemical analysis of sections of buccal mucosa revealed a dramatic increase in the number of class II+ DC within both the epithelium and underlying dermis of FL-treated mice (Fig. 6f) compared with PBS-treated control mice (Fig. 6e). Analysis of the NP-specific CTL response in FL-treated or PBS-treated mice showed that FL treatment did not affect the

![FIGURE 6. Accumulation of MHC class II-positive cells in the buccal mucosa after NP immunization (a–d) or FL treatment (e and f). Immunoperoxidase staining for MHC class II molecules of cryostat sections of buccal mucosa at 2 h after transepithelial injection of PBS alone (a) and at 2 (b) and 24 h (c) after buccal injection of 30 μg NP. Final magnification, ×400. Semiquantitative analysis of MHC class II+ cells in both epithelium and dermis was conducted by microscope observation of immunohistochemical staining of 10 sections of buccal mucosa from individual mice. Results are expressed as the number of class II-positive cells per square millimeter of tissue (d). e and f, Immunohistochemical staining for MHC class II reveals DC accumulation in both suprabasal layers of the buccal epithelium and underlying dermis in mice treated daily for 9 consecutive days with 10 μg FL (right) compared with PBS-treated mice (left). Final magnification, ×400. Hematoxylin counterstaining.
intensity of the CTL response generated by buccal immunization with a high dose of NP (30 μg), but was able to enhance the NP-specific CTL response induced at a suboptimal dose of 10 μg NP (Fig. 7a). To estimate the effect of FL treatment on the frequency of NP-specific CD8^+ T cells, ELISPOT analysis of NP-specific IFN-γ-producing cells was conducted after in vitro overnight restimulation of spleen cells with P815-NP transfectant. As previously reported for hapten (15), the NP-specific IFN-γ-producing cells obtained after in vitro restimulation of splenic effectors with class-II negative P815-NP transfectant comprised only CD8^+ T cells. No spot could be detected if spleen cells were restimulated with untransfected P815 cells or with spleen cells from naive mice with or without NP stimulation in vitro. In control PBS-treated mice, buccal immunization with 10 μg NP induced a frequency of 15 IFN-γ-producing cells/10^6 cells in the spleen. FL-treated mice exhibited a 6-fold increase in the number of NP-specific IFN-γ-producing cells (Fig. 7b).

NP-pulsed BM-DC induce in vivo priming of NP-specific effector T cell

We next examined whether DC could process and present NP on MHC class I molecules and could induce in vivo priming of specific T cells. Day 7 BM-DC were pulsed in vitro with 100 μg NP or BSA and adoptively transferred s.c. to naive mice. On day 10 after immunization, spleen cells were restimulated overnight with either P815-NP or P815 cells as controls, and the number of NP-specific IFN-γ-producing cells was determined by ELISPOT assay. Adoptive transfer of NP-pulsed BM-DC into naive mice induced a frequency of 10–32 NP-specific IFN-γ-producing cells/10^6 cells in the spleen (Fig. 8), indicating that DC could present NP in vivo and activate NP-specific T cell effectors.

Discussion

This study demonstrates that transepithelial delivery of recombinant MV NP via buccal mucosa LC can induce, after a single immunization, the development of NP-specific MHC class I-restricted cytotoxic CD8^+ T cells as well as NP-specific Abs. In vivo priming of NP-specific immune responses could be achieved by either topical application of NP or transepithelial injection of the protein through the buccal mucosa in the absence of adjuvant.

The CTL response primed by buccal immunization with NP is mediated by Ld-restricted CD8^+ T cells, which recognize the dominant Ld epitope NP_{281–289}, similarly to CTL induced by s.c. injection of NP or by tail scarification with a recombinant VV encoding NP (VV-NP) (27). This suggests that the same MHC class I-processing pathway of NP operates in APC of buccal mucosa and skin. Moreover, the CTL response induced by buccal immunization with NP is able to protect mice against a lethal intracerebral challenge with CDV, which shares with MV the unique NP_{281–289} Ld epitope. Previous studies have shown that vaccination with VV-NP or with a VV recombinant encoding the NP_{281–289} inserted into the gene encoding for CD36 generated NP-specific CTL and completely protected mice against CDV challenge; alternatively, a VV-NP recombinant that has a mutation in the consensus sequence of NP_{281–289} did not induce CTL and was unable to protect mice against CDV challenge (27). We observed that all mice immunized by buccal injection of NP survived a lethal CDV challenge.

That the NP-specific immune response generated after buccal immunization resulted from local Ag uptake by APC in the buccal mucosa rather than from Ag access to gut-associated lymphoid tissues or bronchus-associated lymphoid tissues is supported by several observations. Intragastric administration of the highest dose of NP was inefficient at inducing a specific CTL response, probably due to gastric degradation of the protein at low pH. In addition, on day 10 after buccal NP application or injection,
NP-specific IgG-producing cells were found only in cervicomandibular lymph nodes draining the buccal mucosa and not in spleen or lung. Although Ag loading on APC in bronchus-associated lymphoid tissues may occur to some extent after topical NP immunization, the similar CTL dose-response observed after topical and transepithelial immunization indicate that local uptake of NP in the buccal mucosa is sufficient to induce CTL priming. These data indicate that buccal immunization resulted from NP transport from the buccal mucosa via afferent lymph to draining cervicomandibular lymph nodes for local priming of both B and T cells.

The contribution of DC in their natural environment as the major APC responsible for cross-priming MHC class I-restricted CD8+ CTL in vivo is supported by several observations. Treatment with FL, a growth factor that dramatically expands DC in both lymphoid organs as well as in epithelial tissues, including lamina propria of the intestine (28–30) and dermal skin (31)-induced accumulation of class II DC in the buccal mucosa. This resulted in enhanced NP-specific T cell priming at a suboptimal dose of NP, as revealed by the increase in both NP-specific CTL response and NP-specific IFN-γ-producing CD8+ T cells. This observation is reminiscent of previous studies showing that in vivo expansion of intestinal DC by FL enhances cholera toxin-specific intestinal and serum IgA response and protection against cholera toxin (32). Our data document that FL can also potentiate anti-viral CD8+ CTL cross-priming elicited at mucosal surfaces and that adoptive transfer of NP-pulsed BM-DC could prime NP-specific IFN-γ-producing T cells in naive mice. Finally, the efficiency of in vivo priming of a specific CD8+ T cell response after a single buccal immunization with NP at doses down to 100 ng in the absence of adjuvant is compatible with the well-documented efficiency of DC to prime naive T cells.

Interestingly, NP immunization (by buccal application or injection) induced a rapid and transient recruitment of strongly MHC class II+ cells in the buccal mucosa. The recruited cells exhibited morphological features characteristic of DC and were localized primarily in the buccal dermis and in suprabasal layers of the buccal epithelium, as observed following FL treatment. About 50% of the cells accumulating in the buccal dermis expressed CD11b+, indicating their myeloid origin. The observation that both class II+ and CD11b+ cells decreased to initial numbers by 24 h following NP immunization suggested that the recruited cells have migrated to the draining lymph nodes. These findings are reminiscent of previous studies showing that haptens topically applied to the buccal mucosa induce in 2 h local recruitment of DC, which can be recovered 24 h later from draining cervicomandibular lymph nodes as hapten-presenting cells (13, 16). Whether NP acts by inducing the release by epithelial cells and/or endothelial cells in situ of chemokines or proinflammatory cytokines with the ability to promote extravasation of circulating monocytes and/or DC precursors into the dermis and recruitment of DC into the epithelium remains to be determined. In this respect, recent studies demonstrated a selective role of macrophage inflammatory protein-3α secreted by keratinocytes in the recruitment of LC precursors into the skin in normal (33) and inflammatory conditions (34). In addition, transgenic mice overexpressing MCP-1 under the control of the keratin promoter exhibit local accumulation of cells with dendritic morphology in the basal layer of the epidermis (35).

The mechanisms of NP internalization involved in cross-presentation of NP onto MHC class I molecules, which accounts for the efficient CTL response induced by NP, are unclear at present. DC can efficiently transport exogenous Ags into the endogenous class I pathway by macropinocytosis (36) or by receptor-mediated endocytosis (37–39). Both pathways could be involved in class I cross-presentation of recombinant NP. Indeed, in agreement with Fooks et al. (40), we observed that recombinant NP could self-assemble into nucleocapsid-like structures (similar to those described for MV nucleocapsids; Ref. 41). NP particles have also been identified in human DC after in vitro pulse with the recombinant protein (B. Dubois, personal communication). Alternatively, NP, which has been shown to bind to FcγR (18), could also access the MHC class I pathway by FcγR-mediated internalization, which was demonstrated to concomitantly promote maturation of DC and MHC class I-restricted presentation after immune complex internalization (37). It is possible that the potent immunogenicity of NP in vivo is due to its ability to induce DC activation and/or maturation either directly or via triggering the release by epithelial cells of proinflammatory signals, thus by-passing the need of adjuvant and CD4+ T cell help. Indeed, DC activation is a prerequisite for induction of specific class I-restricted CTL responses in vivo and generally requires CD4+ Th cells, which activate DC through CD40 cross-linking (42). However, recent studies showed that DC maturation induced by T cell-independent stimuli, such as LPS, are sufficient to induce specific CTL in mice depleted of CD4+ T cells (43). In the case of strong inflammatory viruses or bacteria, DC can apparently become activated in a CD4+ Th cell-independent way (44–46).

Generation of class I-restricted CTL by buccal immunization is not limited to NP, but can also be induced with several types of nonreplicating Ags. These include haptens, which covalently bind to self proteins, are processed in the MHC class I pathway, and induce tissue inflammation (13); recombinant plasmid DNA with immunostimulatory CpG motifs (14); and invasive recombinant adenylate cyclase toxin from Bordetella pertussis, containing a class I epitope of lymphocytic choriomeningitis virus (47) (N. Chart et al., unpublished observations). Interestingly, the common feature of these Ags, linked to their ability to promote potent Th1 and Tc1 responses reminiscent of that of NP, is their intrinsic adjuvant and/or proinflammatory property (48–53). Buccal DC recruitment, induced by NP, haptens, and adenylate cyclase of B. pertussis, seems to be a hallmark of these Ags associated with their potent immunogenicity. Thus, foreign proteins with unique ability to stimulate innate immunity may lead to efficient CTL responses when delivered topically or intradermally via DC of the buccal mucosa.

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