Eye Mucosa: An Efficient Vaccine Delivery Route for Inducing Protective Immunity

Kyoung Yul Seo, Soo Jung Han, Hye-Ran Cha, Sang-Uk Seo, Joo-Hye Song, So-Hyang Chung and Mi-Na Kweon

*J Immunol* 2010; 185:3610-3619; Prepublished online 13 August 2010; doi: 10.4049/jimmunol.1000680

http://www.jimmunol.org/content/185/6/3610

References

This article cites 43 articles, 19 of which you can access for free at: http://www.jimmunol.org/content/185/6/3610.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Eye Mucosa: An Efficient Vaccine Delivery Route for Inducing Protective Immunity

Kyoung Yul Seo,*† Soo Jung Han,*† Hye-Ran Cha,‡ Sang-Uk Seo,† Joo-Hye Song,† So-Hyang Chung,‡ and Mi-Na Kweon†

The external part of the eye shares mucosa-associated common characteristics and is an obvious entry site for foreign Ags. We assessed the potential of eyedrop vaccination for effective delivery of vaccines against viral or bacterial infection in mice. Both OVA-specific IgG Ab and IgA Ab in mucosal compartments were induced by eyedrops of OVA with cholera toxin (CT). Eyedrop vaccination of influenza A/PR/8 virus (H1N1) induced both influenza virus-specific systemic and mucosal Ab responses and protected mice completely against respiratory infection with influenza A/PR/8 virus. In addition, eyedrop vaccination of attenuated Salmonella vaccine strains induced LPS-specific Ab and complete protection against oral challenge of virulent Salmonella. Unlike with the intranasal route, eyedrop vaccinations did not redirect administered Ag into the CNS in the presence of CT. When mice were vaccinated by eyedrop, even after the occlusion of tear drainage from eye to nose, Ag-specific immune responses and protective immunity were induced effectively. Note, eyedrops with OVA plus CT induced organogenesis of the conjunctiva and increased microfold cell-like cells on the conjunctiva-associated lymphoid tissue in the presence of CT. When mice were vaccinated by eyedrop, even after the occlusion of tear drainage from eye to nose, Ag-specific immune responses and protective immunity were induced effectively. Note, eyedrops with OVA plus CT induced organogenesis of the conjunctiva and increased microfold cell-like cells on the conjunctiva-associated lymphoid tissue in the nictitating membrane on conjunctiva, the mucosal side of the external eye. On the basis of these findings, we propose that the eyedrop route is an alternative to mucosal routes for administering vaccines. The Journal of Immunology, 2010, 185: 3610–3619.

A vaccine that induces an immune response by fortifying mucosal immunity is an effective way of targeting the pathogen before infection occurs (1, 2). Mucosal vaccination, in contrast to parenteral vaccination, is of particular interest because it can elicit both systemic and mucosal immune responses, mainly secretory IgA (sIgA) Abs, at the very portal of entry of most infectious pathogens (3). Vaccine development has lagged behind the rapidity of disease propagation in the era of global travel. Mucosal vaccination, which is easy to administer and does not require special training, has become a strategy to thwart new pathogen strains before they become pandemic.

The eye mucosa is a possible route for mucosal vaccine because it is an important entry point for environmental Ags and infectious materials occupying most of the external ocular surface (4–6). The conjunctiva, part of the eye mucosa, has immunologic features in common with other mucosal tissues. The conjunctiva has CD8+ T cells in the epithelium, equal proportions of CD4+ and CD8+ T cells, B cells, and mast cells in the lamina propria, and dendritic cells (DCs) and Langerhans cells (7, 8). As such, the conjunctiva is part of the mucosal barrier that is exposed to the external environment and shares many common immunologic features of other mucosal compartments. Previous studies showed successful protection by eyedrop vaccination in avian and bovine models (9, 10). However, the underlying mechanism of the induction of acquired immune responses and the systematic comparison of conjunctival and intranasal (i.n.) routes are not yet elucidated (11–13).

In this study, we assessed whether eyedrop administration on the eye mucosa can induce Ag-specific immune and protective efficiency. Eyedrop administration of a prototype protein Ag plus cholera toxin (CT) induced a broad range of immune responses in both mucosal and systemic tissues. In addition, eyedrop vaccination of influenza A/PR/8 virus and recombinant Salmonella strains protected mice against respiratory challenge of influenza virus and oral challenge of Salmonella, respectively. In contrast to i.n. administered vaccines, we showed that eyedrop vaccination poses no risk of Ag redirection to the CNS in the presence of CT. On the basis of our findings, we propose that the eye mucosa is a good candidate for mucosal vaccine delivery for inducing protective immunity and theoretically a safe alternative for vaccine delivery targeting viral or bacterial infection.

Materials and Methods

Mice

Specific pathogen-free BALB/c mice, aged 6–10 wk, were purchased from Charles River Laboratories (Orient Bio, Sungnam, Korea). CCR6−/− mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME), polymeric IgR (plgR)−/− mice were kindly provided by Dr. Masanobu Nanno (Yakult Central Institute for Microbiological Research, Tokyo, Japan). Dr. Kazuhiko Yamamoto (University of Tokyo, Tokyo, Japan) generously made available the OVA-TCR transgenic mice (DO11.10) on a BALB/c background expressing OVA epitope (323–339), and Dr. Martin Lipp (Max Delbruck Center for Molecular Medicine, Berlin, Germany) generously provided the CCR7−/− mice on a C57BL/6 background. All mice were maintained in the experimental animal facility under specific pathogen-free conditions at the International Vaccine Institute (Seoul, Korea) and received sterilized food (Certified Diet MF;
Oriental Yeast, Osaka, Japan) and filtered tap water ad libitum. All animal experiments were approved by the Animal Research Committee of the International Vaccine Institute (Seoul, Korea).

**Immunization**

Prior to experimental manipulation, mice were anesthetized by i.p. injection of ketamine (100 mg/kg body weight) and xylazine hydrochloride (10 mg/kg body weight). For conjunctival immunization, 100 μg OVA (Sigma-Aldrich, St. Louis, MO) and 2 μg CT (List Biological Laboratories, Campbell, CA) were suspended in 5 μl PBS and inoculated into the conjunctival sac by micropipette. In some experiments, mice were immunized with 0.1 × LD50 (500 PFU) live A/PR/8 virus [APuerto Rico8/34 (H1N1)] or recombinant attenuated Salmonella enterica serovar Typhimurium [Δ9241 ΔµaB1516 ΔµaB232 Δαda16 Δα Buddha23 ΔαI918; araCPBADlacI(ATEG)]TT containing pYA3802: 1 × 10^7 CFU] (14, 15) suspended in 5 μl PBS.

**Sample collection**

Serum was obtained by retro-orbital bleeding. Tear-wash samples were obtained by lavaging with 10 μl PBS per eye. Saliva was obtained following i.p. injection of mice with pilocarpine (500 mg/kg body weight; Sigma-Aldrich). Fecal extract was obtained by adding weighed feces to PBS containing 0.1% sodium azide. The feces were mixed by vortexing and centrifuged, and the supernatants were collected for assay. Vaginal wash samples were collected by lavage with 100 μl PBS. After the mice were sacrificed, nasal wash samples were obtained by flushing 100 μl PBS through the anterior (oral) entrance of the nasal passages (NPs) using a pipette.

**ELISA for detection of Ag-specific Ab**

ELISA plates (Falcon, Franklin Lakes, NJ) were coated with OVA (100 μg/ml) or inactivated A/PR/8 (5 μg/ml) or LPS (1 μg/ml) in PBS and incubated overnight at 4°C. Blocking was done with 1% BSA (Sigma-Aldrich) in PBS, and 2-fold serially diluted samples were applied to plates. HRP-conjugated goat anti-mouse IgG or IgA Ab (Southern Biotechnology Associates, Birmingham, AL) was added to each well and incubated overnight at 4°C. For color development, tetramethylbenzidine solution (Moss, Pasadena, MD) was used. Then, plates were measured at 450 nm on an ELISA reader (Molecular Devices, Sunnyvale, CA) after addition of a conjugate. To detect OVA-specific IgA Ab levels, plates were coated with OVA (100 μg/ml) and goat anti-pIgR (R&D Systems, Minneapolis, MN) and HRP-conjugated rabbit anti-antibody IgG (Southern Biotechnology Associates) were used as detection Abs.

**In vitro T cell proliferation assay**

Following eyedrop vaccination with a mixture of OVA and CT, DCS were isolated from submandibular lymph nodes (SMLNs), as well as jugular, mediastinal, axillary, inguinal, and iliac lymph nodes (LNs). CD4^+KJ1.26^- T cells isolated from DO11.10 mice were labeled with CFSE (Invitrogen, Carlsbad, CA) for 15 min at 37°C and washed several times in PBS. The purified DCS (3 × 10^5 cells/well) were cocultured with CD4^+KJ1.26^- T cells (2 × 10^5 cells/well) in the presence of OVA peptide (OVA323–339) for 2 d at 37°C. CFSE fluorescence in each tissue was analyzed by FACScalibur (BD Biosciences, Franklin Lakes, NJ). To assess OVA-specific T cell proliferation in vitro, CD4^+ T cells isolated from SMLNs of vaccinated mice and CD3^+ T cell-depleted splenocytes were prepared from naive mice. CD4^+ T cell- (2 × 10^5 cells/well) and CD3^+ T cell-depleted splenocytes (2.5 × 10^5 or 1.25 × 10^5 cells/well) were cocultured in 10% FBS containing RPMI 1640 for 3 d in the presence of OVA peptide. [H]Thymidine incorporation was measured by scintillation counter (Perkin Elmer, Waltham, MA).

**Ag uptake in situ**

S. typhimurium PhoPc strain transformed with the pKK GFP plasmid and GFP-expressing Yersinia pseudotuberculosis were prepared by the method described (18). GFP-expressing bacteria (5 × 10^5 CFU) were suspended in 5 μl PBS and inoculated into the conjunctival sac of anesthetized mice and incubated in situ for 10 min. Then, whole conjunctiva, including nictitating membranes, was removed and extensively washed with cold PBS and RPMI medium including gentamicin (50 μg/ml). Conjunctival tissues were fixed in 4% paraformaldehyde and dehydrated in graded ethanol solution. Dehydrated tissues were critical point-dried with CO2, sputter-coated, and observed with a JSM-5410LV scanning electron microscope (JEOL, Tokyo, Japan). For TEM analysis, tissues were fixed with 4% paraformaldehyde in PBS were immersed in 0.3% H2O2, diluted in methanol for 30 min to block endogenous peroxidase, incubated with 20 μg/ml UEA-1-HRP in PBS for 1 h, and then stained with 3,3’-diaminobenzidine tetrahydrochloride (DAB). After staining, the tissues were fixed overnight with 2% glutaraldehyde in 100 mM sodium phosphate buffer (pH 7.3) at 4°C. The tissues were subjected to TEM analysis (JEM 1010, JEOL).

**Results**

Significant induction of Ag-specific Ab responses by eyedrop administration

To assess the efficacy of eyedrop administration for inducing systemic and mucosal Ab responses, BALB/c mice were immunized three times at 1-wk intervals. At 1 wk after final immunization, the
levels of Ag-specific Abs and the numbers of Ab-secreting cells (ASCs) were measured by ELISA and ELISPOT, respectively. Groups of BALB/c mice given OVA (100 μg) plus CT (2 μg) or OVA alone or PBS by drops on both eyes and i.n. every week (three times). OVA-specific Ab levels were measured in serum and in various mucosal secretions 1 wk after final vaccination by ELISA. B. Mononuclear cells from SP, SMLN, NP, LG, conjunctiva, and SMG were prepared from eyedrop-immunized mice given OVA plus CT or PBS three times at 1-wk intervals. ELISPOT assay was adopted to address numbers of OVA-specific ASCs. C. In plgR−/− mice of B6 background, OVA-specific IgA Ab in mucosal secretions were compared with those in wild-type B6 mice following eyedrop vaccination with OVA plus CT. D. Ratio of OVA-specific sIgA Ab (OD) in total IgA Ab (OD) in tear and vaginal wash samples (×200 dilution). *p < 0.05; **p < 0.01; ***p < 0.001 versus PBS-vaccinated group. LG, lacrimal gland; n.d., not detected; SMG, submandibular gland; SP, spleen.

FIGURE 1. Eyedrop administration of OVA plus CT resulted in both systemic and mucosal Ab responses. A. Groups of BALB/c mice received OVA (100 μg) plus CT (2 μg) or OVA alone or PBS by drops on both eyes and i.n. every week (three times). OVA-specific Ab levels were measured in serum and in various mucosal secretions 1 wk after final vaccination by ELISA. B. Mononuclear cells from SP, SMLN, NP, LG, conjunctiva, and SMG were prepared from eyedrop-immunized mice given OVA plus CT or PBS three times at 1-wk intervals. ELISPOT assay was adopted to address numbers of OVA-specific ASCs. C. In plgR−/− mice of B6 background, OVA-specific IgA Ab in mucosal secretions were compared with those in wild-type B6 mice following eyedrop vaccination with OVA plus CT. D. Ratio of OVA-specific sIgA Ab (OD) in total IgA Ab (OD) in tear and vaginal wash samples (×200 dilution). *p < 0.05; **p < 0.01; ***p < 0.001 versus PBS-vaccinated group. LG, lacrimal gland; n.d., not detected; SMG, submandibular gland; SP, spleen.

FIGURE 2. SMLNs drain directly after eyedrop vaccination. A. DCs were isolated from each LN following eyedrop vaccination with OVA plus CT and cocultured with CFSE-labeled CD4+ T cells from OVA-specific TCR transgenic mice. B. CD4+ T cells were prepared from SMLNs of eyedrop-immunized mice given OVA plus CT or PBS three times at 1-wk intervals and cocultured with CD3+ T cell-depleted splenocytes (APCs) from naive mice. [3H]Thymidine incorporation was shown as cpm. C. CCR6 signals are crucial for induction of OVA-specific IgA Ab responses in mucosal secretions following eyedrop application. Results are representative of three independent experiments, with three mice in each experimental group. *p < 0.05; **p < 0.01; ***p < 0.001 versus PBS-vaccinated group.
indicate that most Ag-specific IgA Abs elicited in the mucosal compartments by eyedrop are in the dimeric form of slgA. Taken together, eyedrop vaccination elicited Ag-specific IgG and slgA Abs in systemic and mucosal tissues effectively, and the increased Ab levels were similar to those induced by i.n. administration.

Ags drain to SMLNs and IgA response is dependent on CCR6 rather than CCR7 in eyedrop immunization

To determine the anatomic location of draining LNs, where Ag is presented to T cells following eyedrop administration, DCs were harvested from SMLNs and from jugular, mediastinal, axillary, and sublingual lymph nodes of mice vaccinated by eyedrop. Representative results are shown in Figure 3A. As outlined in the section on Eyedrop Vaccination, IgA Ab responses were measured by ELISA in serum and in various mucosal secretions 2 wk after vaccination. *p < 0.01; **p < 0.001 compared with findings in the PBS-vaccinated group. B, At 2 wk after eyedrop administration, mice were challenged i.n. with 10 × LD₅₀ of influenza A/PR/8 virus. Body weight and survival rates were monitored daily. C, At 3 d after i.n. challenge, viral titers in bronchial alveolar lavage fluid were measured by plaque assay. **p < 0.001 compared with the PBS group. D, Body weight change was monitored daily after challenge with 10 × LD₅₀ of influenza A/PR/8 virus by eyedrop or i.n. route without any primary vaccination. Results are representative of three independent experiments, with five mice in each group.

**FIGURE 4.** Eyedrop vaccination with attenuated S. typhimurium vaccine strain protects mice against an LD of virulent Salmonella strain (UK-1). A, Levels of LPS-specific Ab responses were measured by ELISA in systemic and mucosal secretions 4 wk after a single eyedrop with attenuated S. typhimurium vaccine strain. *p < 0.05; **p < 0.01; ***p < 0.001, compared with findings in the PBS-treated control group. B, Daily body weights and survival rates after oral challenge with Salmonella strain (UK-1). Results are representative of two independent experiments, with five mice in each group.
inguinal, mesenteric, or iliac LNs of BALB/c mice 24 h after treatment with a single dose of OVA plus CT. Then, each DC was cocultured with CD4+KJ1.26+ cells isolated from OVA-TCR transgenic mice. Only CD4+KJ1.26+ cells cocultured with DCs isolated from SMLNs showed significant proliferation of CD4+ T cells when compared with the CD4+ T cell alone group. The CD4+KJ1.26+ cells cocultured with DCs from other LNs, including jugular, mediastinal, axillary, inguinal, mesenteric, and iliac, did not show any significant proliferation, with DCs from other LNs, including jugular, mediastinal, axillary, inguinal, mesenteric, and iliac, did not show any significant proliferation (Fig. 2A). To address OVA-specific CD4+ T cell proliferation, CD4+ T cells were isolated from SMLNs of naive and eyedrop-vaccinated mice with OVA plus CT three times each week and cocultured with CD3+ T cell-depleted splenocytes from naive mice (Fig. 2B). Higher levels of proliferation were shown in the CD4+ T cells from eyedrop-vaccinated mice than in those from wild-type mice. These OVA-specific proliferations were more enhanced when the CD4+ KJ1.26+ cells cocultured with the CD4+ T cell alone group. The CD4+ KJ1.26+ cells cocultured with DCs from other LNs, including jugular, mediastinal, axillary, inguinal, mesenteric, and iliac, did not show any significant proliferation (Fig. 2A). To address OVA-specific CD4+ T cell proliferation, CD4+ T cells were isolated from SMLNs of naive and eyedrop-vaccinated mice with OVA plus CT three times each week and cocultured with CD3+ T cell-depleted splenocytes from naive mice (Fig. 2B). Higher levels of proliferation were shown in the CD4+ T cells from eyedrop-vaccinated mice than in those from wild-type mice. These OVA-specific proliferations were more enhanced when professional APCs were added (Fig. 2B). Recent studies by ourselves and others indicate that different chemokine–chemokine receptors are involved in eliciting efficient Ag-specific systemic and mucosal immune responses (including T and B cells) by i.n., oral, or sublingual routes (17, 21–23). We thus investigated whether OVA-specific Ab responses could be dependent on CCR6 or CCR7 signaling in eyedrop vaccination by analyzing the OVA-specific Ab titers in both systemic (i.e., serum) and mucosal compartments following eyedrop administration of OVA plus CT using CCR6+ and CCR7+ mice. Of note, there were significantly lower levels of OVA-specific IgA Abs in mucosal compartments of CCR6+ mice than in wild-type and CCR7+ mice (Fig. 2C). These data demonstrate that induction of mucosal IgA Ab responses by eyedrop are tightly regulated in a CCR6-dependent manner, but not by CCR7.

**Eyedrop vaccination of live A/PR/8 virus protects mice against lethal challenge with influenza virus**

To address the efficacy and safety of the eyedrop route for delivery of live influenza virus vaccine, groups of mice were administered live A/PR/8 virus (0.1 × 10^5 PFU) without any boosting. This dose elicited much higher levels of virus-specific IgG Abs in serum and IgA Abs in mucosal secretions (e.g., tears, lung wash, nasal wash, and saliva) of mice given A/PR/8 than was observed in those given PBS (Fig. 3A). Of interest, eyedrop immunization with live A/PR/8 virus resulted in no body weight loss and 100% protection (Fig. 3B) and cleared influenza virus efficiently from the bronchial alveolar lavage fluid (Fig. 3C) against lethal i.n. challenge with A/PR/8 virus (20 × 10^3 PFU). We also compared the body weight changes between eyedrop-vaccinated and i.n.-vaccinated mice given live A/PR/8 virus (0.1 × 10^5 PD50; 500 PFU). In contrast to the i.n. route, mice given vaccine by eyedrop did not lose body weight (Fig. 3D). Overall, eyedrop vaccination with live A/PR/8 virus is safer than i.n. inoculation and is highly effective in protecting mice against lethal respiratory challenge with influenza virus.

**Eyedrop administration of attenuated Salmonella vaccine strain protects mice against lethal challenge with Salmonella**

To further investigate the efficacy of eyedrop vaccination against bacterial infection, we immunized mice by a single eyedrop using...
At 4 wk after immunization, levels of LPS-specific IgG and IgA Abs in the serum and IgA Abs in the mucosal secretions were significantly increased in mice vaccinated by eyedrop with RASV, compared with those given PBS (Fig. 4A). We then challenged groups of vaccinated mice with a lethal dose of virulent Salmonella strain (UK-1, $1 \times 10^7$ CFU) by the oral route. Eyedrop administration with RASV resulted in 100% survival without any loss of body weight against lethal oral challenge with Salmonella (Fig. 4B). When these results are considered together, eyedrop vaccination may also be an effective and innovative mucosal vaccine delivery system against enteric infectious bacterial pathogens.

The trafficking of vaccine Ag into the CNS following i.n. administration with CT or the heat-labile enterotoxin (LT) as adjuvant raises safety concerns (24, 25) and poses a serious obstacle to the clinical use of this route for vaccine delivery. Thus, we sought to determine if eyedrop vaccination would be similarly limited by Ag trafficking to the CNS. We administered acridinium-labeled OVA plus CT to groups of mice via either the eyedrop or the i.n. route and obtained nasal washes at 30, 60, 120, and 240 min after inoculation. The levels of acridinium in the nasal wash specimens were high in the i.n. group and significantly lower in the eyedrop group at all points.

**FIGURE 6.** M and goblet cells on the nictitating membrane of conjunctiva following eyedrop vaccination. A, CALT appeared in the nictitating membrane of mice after eyedrop vaccination with OVA plus CT three times 1 wk apart (a, d) or naive (b) or after one-time (c) eyedrop vaccination (H&E, original magnification $\times 100$ (a), $\times 200$ (b–d). B, Two different cell types were induced on the nictitating membrane of mice that received OVA plus CT by eyedrop (c, d) or PBS alone (a, b). Some cells formed clusters with more rounded tops and straighter microvilli (arrowheads in b, d, f); another type of M cell-like cells did not make clusters and had more irregular and shorter microvilli (arrows in b, d, e).

**Eyedrop-administered Ag passes into the nasal cavity minimally and does not enter the CNS**

The trafficking of vaccine Ag into the CNS following i.n. administration with CT or the heat-labile enterotoxin (LT) as adjuvant raises safety concerns (24, 25) and poses a serious obstacle to the clinical use of this route for vaccine delivery. Thus, we sought to determine if eyedrop vaccination would be similarly limited by Ag trafficking to the CNS. We administered acridinium-labeled OVA plus CT to groups of mice via either the eyedrop or the i.n. route and obtained nasal washes at 30, 60, 120, and 240 min after inoculation. The levels of acridinium in the nasal wash specimens were high in the i.n. group and significantly lower in the eyedrop group at all points.
measurement times (Fig. 5A). At 24 h after inoculation, i.n. administration of acridinium-labeled OVA resulted in the accumulation of Ag in lung and olfactory bulbs (Fig. 5B). In contrast, acridinium-labeled OVA was undetectable in both sites following eyedrop administration, demonstrating that this route did not redirect Ag into the CNS. When the same experiment was performed with acridinium-labeled CT alone, eyedrop administration did not redirect CT into the CNS, whereas i.n. administration did (Fig. 5B).

To directly prove the role of eye mucosa in inducing systemic and mucosal immune responses, we blocked the passage of eyedrop Ag into nasal mucosa by suturing four nasolacrimal duct puncta, blocking the draining route from eye to nose. To confirm the blockade of passage into the nasal mucosa, acridinium levels were determined in the NP 24 h following eyedrop of acridinium-labeled OVA plus CT. As expected, significantly less acridinium was found in the sutured mice compared with those not sutured (Fig. 5C). Of note, eyedrop inoculation of sutured mice induced levels of IgA Abs in the serum and mucosal secretions identical to those in the nonsutured mice (Fig. 5D). These results strongly suggest that effective induction of T and B cell responses by eyedrop administration is completely independent of the inductive capacity of the nasal cavity.

Organogenesis of conjunctiva-associated lymphoid tissue and increased M cell- and goblet cell-like cells on the nictitating membrane of eye mucosa after eyedrop immunization

To evaluate the structure of eye mucosa as an Ag delivery site involving protective immunity by eyedrop vaccination, we examined whole-eye specimens by histologic staining and electron microscopic methods. The mucosal surfaces of Ag-treated eyes showed lymphoid tissues in the nictitating membrane (the third eyelid; homologous with the plica semilunaris in humans) (Fig. 6A). The development of conjunctiva-associated lymphoid tissue (CALT) during eyedrop immunization was observed in a time-dependent manner (Fig. 6Ab–d). In this tissue, CALT is one of the peripheral lymphoid organs that can contain M cells with Ag sampling function. Scanning electron microscopy demonstrated increased numbers of two different types of cells on the nictitating membrane after eyedrop administration of OVA plus CT (Fig. 6B, 6D), when compared with PBS-treated mice (Fig. 6Ba, 6Bb). We found epithelial cells having depressed surfaces with irregular and short microvilli (Fig. 6Bd, arrow, and 6Be) and clusters of cells with more rounded tops and straighter microvilli, which were observed only in conjunctiva goblet cells (Fig. 6Bd, arrowhead, and 6Bf) (26, 27).

TEM analysis after the binding of HRP-conjugated UEA-1 Ab (DAB reacted) showed a typical morphology of UEA-1+ conjunctival epithelial cells following eyedrop administration of OVA plus CT (Fig. 7A, 7B). Of note, M cells had more irregular and coarse UEA-1+ microvilli (Fig. 7C, 7D) with infiltrating mononuclear cells beneath the cell membrane (Fig. 7C, arrowheads), much like a feature of pocket lymphocytes in intestinal M cells (18, 28). In addition, clustered cells had straighter microvilli, and goblet cells possessed many vesicles in their cytoplasm (Fig. 7E, 7F).

FIGURE 7. M and goblet cells on the nictitating membrane of conjunctiva following eyedrop vaccination. TEM analysis with UEA-1-HRP Ab (DAB reacted). UEA-1+ M cells show longer and coarser microvilli (C, D) than do UEA-1+ epithelial cells (A, B) and the presence of infiltrating mononuclear cells (arrowheads in C). Microvilli of goblet cells are straighter than those of M cells, and goblet cells have many vesicles within (E, F). Original magnification ×3,000 (A, E), ×4,000 (C), ×10,000 (F), ×20,000 (B, D).

UEA-1+ M cells can sample and internalize the rSalmonella-GFP

Because expression of α(1, 2) fucose is a hallmark of murine M cells, lectin UEA-1 possessing affinity for α(1, 2) fucose is routinely used to detect such cells in mice (29). UEA-1 also reacts to goblet cells; however, these cells possess strong affinity to epithelial cell-specific lectin, such as WGA (18). Using confocal image analysis of whole-mount murine conjunctiva stained with TRITC-conjugated...
UEA-1 (red) and FITC-labeled WGA (green) Ab, we found UEA-1⁺ M cell-like cells in conjunctival epithelium of nictitating membrane. A, Confocal image analysis demonstrates that M cells (UEA-1⁺WGA⁺, arrow), goblet cells (UEA-1⁺WGA⁺, arrowheads), and epithelial cells (UEA-1⁺WGA⁻) were increased in the nictitating membrane of conjunctiva of OVA plus CT-treated mice (c, d) when compared with PBS-treated mice (a, b). Samples were whole-mount (a, c) or frozen sections (b, d). B, After eyedrops were applied with GFP-expressing Salmonella typhimurium (a, b) or Y. pseudotuberculosis (c–e), whole-mount (a–d), and frozen sections (e) of conjunctiva of nictitating membrane were prepared and stained with UEA-1-TRICT. Original magnification ×400.

FIGURE 8. Sampling and internalizing of bacteria by UEA-1⁺ M cell-like cells in conjunctival epithelium of nictitating membrane. A, Confocal image analysis demonstrates that M cells (UEA-1⁺WGA⁺, arrow), goblet cells (UEA-1⁺WGA⁺, arrowheads), and epithelial cells (UEA-1⁺WGA⁻) were increased in the nictitating membrane of conjunctiva of OVA plus CT-treated mice (c, d) when compared with PBS-treated mice (a, b). Samples were whole-mount (a, c) or frozen sections (b, d). B, After eyedrops were applied with GFP-expressing Salmonella typhimurium (a, b) or Y. pseudotuberculosis (c–e), whole-mount (a–d), and frozen sections (e) of conjunctiva of nictitating membrane were prepared and stained with UEA-1-TRICT. Original magnification ×400.

Discussion

Mucosal vaccination has the advantage of producing both sIgA Abs in mucosal compartments and IgG Abs in serum, in contrast to parenteral vaccines, which induce only serum IgG Abs. Such sIgA Ab responses play an important role in protecting against the invading external pathogen on the mucosal surface. No changes in behavior, weight loss, or local inflammation were observed in mice after eyedrop administration. Our results provide the evidence that eyedrop vaccination induces both mucosal and systemic immune responses that it is protective against virus (i.e., influenza) and bacteria (i.e., Salmonella) infections in mice.

To determine the mechanism of vaccine delivery by a novel route, a crucial step is to demonstrate the Ag delivery pathway by sampling cells and the draining lymphoid organs for Ag presentation. The fact that Ag-specific CD4⁺ T cell expansion occurred only in the SMLNs after eyedrop application with OVA plus CT (Fig. 2A) suggests that SMLNs target draining LN after eyedrop vaccination. As a drainage site of Ag from eye, SMLNs were the most commonly mentioned candidate in an earlier study and were suggested as the main priming site of donor Ag in corneal allograft (30). In addition, following posterior chamber injection of Ag, adopted KJ1-26⁺ cells accumulated primarily in the SMLNs within 3 d (31). Further, cells presenting OVA peptide (OVA323–339) in vivo were found only in the SMLN but not in other LN, spleen, or nasal-associated lymphoid tissue after conjunctival application of OVA together with colonization-factor Ag in the mice (32). Together these findings suggest that the corneal conjunctiva and the posterior eye chamber share the common draining SMLN for induction of immunity or tolerance.

These data also suggest that eyedrop-administered vaccine enters through the eye mucosa, reaches the SMLN, and induces effective immunity against a pathogen. However, because of tear drainage from eye to nose, it is important to show that the eyedrop route does not share the characteristics of the i.n. pathway. Our results indicate that the eyedrop route relies on the chemokine receptor CCR6, contrary to CCR7 dependency in the i.n. or sublingual pathways (Fig. 2B) (21, 33). Eyedrop vaccination did not redirect Ag into CNS (Fig. 5B), as reported for i.n. delivery when CT and LT were used as adjuvant, which was considered a possible reason for nerve damage in one clinical trial (24, 25). Normal levels of Ag-specific Ab responses in serum and mucosal secretions were induced by eyedrop vaccination after saturaing four puncta of the nasolacrimal duct (Fig. 5C). Finally, live virus used in 0.1 × LD₅₀ doses for i.n. vaccination caused body weight loss, but eyedrop inoculation had no effect on body weight (Fig. 3B). Therefore, these results imply that eyedrop vaccination is unique and a safer method of mucosal vaccine delivery independent of nasal mucosa.

Nagatake et al. (34) showed that tear duct-associated lymphoid tissues (TALTs), which are located in the lacrimal sac, play a role in the induction of Ag-specific immune response against Ag found on the ocular surface. TALT is the site of ocular Ag uptake by M cell-like cells and also the site for induction of Ag-specific IgA and CD4⁺ cells after ocular immunization. Thus, it is possible that TALT could be one candidate for inductive eyedrop vaccination. In addition to TALT, our study demonstrated, after Ag application to
the conjunctiva by eyedrop vaccination, that lymphoid follicles (i.e., CALT) on the nictitating membranes of murine conjunctiva became sufficiently large to be detectable by microscopy (Fig. 6a, 6d). At steady state, 8% of mice (2 of 25) showed organized CALT in the nictitating membranes of conjunctiva (data not shown). A previous study revealed the existence of follicles in the nictitating membrane of mouse conjunctiva and their characteristic plasticity in terms of size and numbers after conjunctiva OVA challenge (35, 36). Furthermore, Steven et al. (37) also showed induction of CALT in 70% of mice by external application of Chlamydia trachomatis serovar C or a solution of OVA and B subunit of CT. It seems likely that CALT could be another candidate as an inductive site for eyedrop vaccination.

In this study, we also found the existence of M cell-like cells (i.e., UEA-1+WGA cells) in the CALT of the nictitating membrane of mouse conjunctiva. M cells, a unique epithelial cell type specializing in Ag sampling, have been discovered in follicle-associated epithelium of TALT, GALT, and nasal-associated lymphoid tissue (34, 38, 39). The M cell-like cells found in our study after eyedrop application were depressed from adjacent epithelial cells and had irregular and longer microvilli (Figs. 6b, 7). Others have reported the presence of M cells in conjunctival mucosa in several species, including rabbit, guinea pig, and canine (26, 40–42). M cells in the guinea pig conjunctiva were located in follicle-associated epithelium and showed various sized microvilli (100 nm–1 μm), in contrast to epithelial cells (350 nm) (26, 43). These characteristics were also found in M cells in rabbit conjunctiva, which had longer and more irregular microvilli than did epithelial cells (41). Further, M cells in the conjunctiva of both guinea pig and rabbit had properties for endocytosis and/or transcytosis. In contrast, canine M cells had shorter and attenuated microvilli compared with M cells from the other two species (40).

In our present study, microvilli of M cells in murine conjunctiva were longer and more irregular than those of the adjacent epithelial cells, suggesting more similarity to rabbits and guinea pigs than to canines. Most importantly, UEA-1+WGA-M cell-like cells in the nictitating membrane of mouse conjunctiva are able to sample and internalize Ag, such as Y. pseudotuberculosis, asahi, Y. asahi, Y. hasegawa, y. sato, S. shimada, M. nanno, M. yatsuoka, M. ohwaki, etc. 2002. Protection against influenza virus infection in polymeric Ig receptor knockout mice immunized intranasally with adjuvant-combined vaccines. J. Immunol. 168: 2930–2938.


