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

Why *The JI*?
- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

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 in serum 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 systemic IgG and mucosal IgA Abs could be induced effectively. Of note, eyedrops with OVA plus CT induced organogenesis of the presence of CT. When mice were vaccinated by eyedrop, even after the occlusion of tear drainage from eye to nose, Ag-specific systemic IgG and mucosal IgA Abs could be induced effectively. Of note, eyedrops with OVA plus CT induced organogenesis of conjunctiva-associated lymphoid tissue 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 (slgA) 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

*Institute for Vision Research, Department of Ophthalmology, Yonsei University College of Medicine; †Mucosal Immunology Section, International Vaccine Institute; and ‡Department of Ophthalmology and Visual Science, College of Medicine, The Catholic University of Korea, Seoul, Korea

Address correspondence and reprint requests to Dr. Mi-Na Kweon, Mucosal Immunology Section, International Vaccine Institute, Seoul National University Research Park, Kwanak-Gu, Seoul, Korea 151-818. E-mail address: minkweon@ivi.int

Abbreviations used in this paper: ASC, Ab-secreting cell; CALT, conjunctiva-associated lymphoid tissue; CT, cholera toxin; DAB, 3,3′-diaminobenzidine tetrahydrochloride; DC, dendritic cell; i., intranasal(i.y); I.G, lacrimal gland; LN, lymph node; n.d., not detected; NP, nasal passage; OB, olfactory bulb; pIgR, polymeric IgR; RASV, recombinant attenuated Salmonella typhimurium vaccine; slgA, secretory IgA; SMG, submandibular gland; SMLN, submandibular lymph node; SP, spleen; TALT, tear duct-associated lymphoid tissue; TEM, transmission electron microscope; TRITC, tetramethylrhodamine isothiocyanate; UEA-1, Ulex europaeus agglutinin; WGA, wheat germ agglutinin.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10/$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1000680

Received for publication February 26, 2010. Accepted for publication July 8, 2010.

This work was supported by a grant from the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare and Family Affairs, Republic of Korea (A084790).

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 (pIgR)−/− mice were kindly provided by Dr. Masanobu Nanno (Yakult Central Institute for Microbiological Research, Tokyo, Japan). Dr. Kazuhiro 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) suspended in 5 μl PBS were dropped weekly for 3 consecutive weeks onto a conjunctival sac by micropipette. In some experiments, mice were immunized with 0.1 × 10^7 CFU of CT (500 PFU) live A/PR/8 virus [APuerto Rico8/34 (H1N1)] or recombinant attenuated Salmonella enterica serovar Typhimurium [p9241 ΔpabA1516 ΔpabB232 ΔdaiA16 ΔtauAD23 ΔvexAI98: areCPBADlacATG] 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) and 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 OVA-specific sIgA Ab levels. To detect OVA-specific sIgA Abs, plates were coated with goat F(ab’)2 anti-mouse IgG, and HRP-conjugated goat anti-mouse IgG (Southern Biotechnology Associates) were used as detection Abs.

**In vitro T cell proliferation assay**

Following eye-drop 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 proliferation 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 naïve mice. CD4^+ T cell (2 × 10^6 cells/well) and CD3^+ T cell-depleted splenocytes (2.5 × 10^6 or 1.25 × 10^6 cells/well) were cocultured in 10% FBS containing RPMI 1640 for 3 d in the presence of OVA peptide. [3H]-Thymidine incorporation was measured by scintillation counter (Perkin Elmer, Waltham, MA).

**Ag tracking**

To determine whether eye-drop vaccination could redirect Ag into the CNS (16, 17), mice were given acridinium-labeled OVA (40 μg) plus CT (2 μg) or acridium-labeled CT (2 μg; List Biological Laboratories) via the i.n. (5 μl for each nostril) or eye route (5 μl for each eye). Then, lung and olfactory bulb were removed 24 h following immunization, as previously described (17). Tissues were weighed, and 200 μl CellLytic MT lysis buffer (Sigma-Aldrich) was added per 10 mg wet weight tissue. Tissues were homogenized and frozen at −20°C. After thawing, the homogenates were centrifuged at 10,000 × g for 10 min, and the supernatants were tested for light activity using the Lmax II384 system ( Molecular Devices).

**Protection assay against influenza virus A/PR/8/34**

At 2 wk after eye drop immunization with 0.1 × 10^7 PFU A/PR/8 virus, anesthetized mice were challenged with live A/PR/8 virus suspension (20 × 10^4 PFU) by the i.n. route. Animals were monitored for weight loss and survival every day for 12 d.

**Protection assay against the wild-type virulent S. typhimurium (UK-1) strain**

Four weeks after eye drop immunization with recombinant attenuated S. typhimurium vaccine (RASV) strain (1 × 10^7 CFU), the virulent UK-1 strain (1 × 10^7 CFU) was given orally for a challenge experiment. Body weight and survival were monitored every day for 8 d.

**M cell staining**

For the preparation of whole-mount staining, the small nictitating membrane of the conjunctiva of naive or immunized mice was fixed in 4% paraformaldehyde and 2% paraformaldehyde in PBS containing 100 mM HEPES for 1 h at reverse transcription. After being washed with PBS, specimens were treated with 1% osmium tetroxide for 1 h at reverse transcription 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 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% paraformaldehyde in 100 mM sodium phosphate buffer (pH 7.3) at 4°C. The tissues were subjected to TEM analysis (JEM 1010, JEOL).

**Electron microscopic evaluations**

Scanning and transmission electron microscopic (TEM) analyses were performed for the characterization of M cells. For scanning electron microscopic analysis, small fragments of the nictitating membrane of the conjunctiva were cleaned of mucus and fixed in 2% glutaraldehyde and 2% paraformaldehyde in PBS containing 100 mM HEPES for 1 h at reverse transcription. After being washed with PBS, specimens were treated with 1% osmium tetroxide for 1 h at reverse transcription 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 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% paraformaldehyde in 100 mM sodium phosphate buffer (pH 7.3) at 4°C. The tissues were subjected to TEM analysis (JEM 1010, JEOL).

**Ag uptake in situ**

S. typhimurium PhoPc strain transformed with the pKK GFP plasmid and GFP-expressing Versinia pseudotuberculosis were prepared by the method described (18). GFP-expressing bacteria (5 × 10^7 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 glycol solution (10, 20, and 30%). The specimens were embedded in O.C.T. compound (Tissue-Tek, Tokyo, Japan) and stored at −80°C until processing. Cryostat sections (5 μm) were fixed in ice-cold acetone and blocked with FcRHIgG mAb (BD Pharmingen, San Diego, CA) in PBS. For lectin staining, tissues were stained with 20 μg/ml tetramethylrhodamine isothiocyanate TRITC-conjugated Ulex europaeus agglutinin (UEA-1) (Vector Laboratories, Burlingame, CA) and FITC-conjugated wheat germ agglutinin (WGA). DAPI (Invitrogen) was used for nucleus staining. Sections were studied by confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

**Data and statistical analyses**

Data were expressed as the mean ± SD, and statistical analyses were done by the t test (Sigma plot program).

**Results**

Significant induction of Ag-specific Ab responses by eye drop administration

To assess the efficacy of eye drop 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 mice given OVA (100 μg/dose) plus CT (2 μg) by eyedrop showed significantly higher levels of OVA-specific IgG and IgA Ab in serum and IgA Ab in mucosal secretions (e.g., tear, nasal, saliva, and vaginal wash) than found in control mice given PBS or OVA alone (Fig. 1A). Levels of OVA-specific IgG and IgA Abs induced by eyedrop were identical to those elicited by i.n. administration with the same dose of OVA plus CT (Fig. 1A). Because some Abs in secretions may originate from plasma by tran-

sudation of Ig, ELISPOT assays were carried out to determine the contribution of the local plasma cell pool after eyedrop immunization. OVA-specific ASCs were counted in cell suspensions from spleen, SMLNs, NP, lacrimal gland, conjunctiva, and subman-
dibular gland 2 wk after the final vaccination. Eyedrop immunization with OVA plus CT elicited higher numbers of OVA-specific IgA ASCs in all tested tissues than did PBS (Fig. 1B). We used plgR−/− mice, in which the transepithelial transport of dimeric IgA is blocked, to measure whether IgA Abs induced by eyedrop are in secretory form (19, 20). As shown in Fig. 1C, OVA-specific IgA Abs were not detectable in tears or in nasal, saliva, and vaginal wash samples of plgR−/− mice, but they produced identical levels of serum IgG and IgA Abs following eyedrop vaccination. Although some IgA Abs were detected in the nasal wash of plgR−/− mice, this was expected because of inevitable blood contamination during nasal wash preparation. To exclude the possibility of nonspecific increase of IgA in mucosal secretions, we determined the ratio of OVA-specific sIgA in total IgA Abs (Fig. 1D). These results

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 sIgA. Taken together, eyedrop vaccination elicited Ag-specific IgG and sIgA Abs in systemic and mucosal tissues effectively, and the increased Ab levels were similar to those induced by i.n. administration.

**FIGURE 3.** Eyedrop administration with live A/PR/8 virus can protect against lethal influenza virus challenge. A, Mice were vaccinated with a single dose of influenza virus A (0.1 × LD₅₀, A/PR8, H1N1), i.n. or by eyedrop. Levels of anti-A/PR/8 virus-specific IgG and 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.
FIGURE 5. Eyedrop-administered Ag passes into the nasal cavity minimally and does not redirect Ags to the CNS. A. Levels of acridinium activity in nasal wash samples at 30, 60, 120, and 240 min after eyedrop or i.n. administration with acridinium-labeled OVA (40 μg) plus CT (2 μg). B, BALB/c mice were immunized with acridinium-labeled OVA plus CT or acridinium-labeled CT via the i.n. route or by eyedrop and sacrificed 24 h later. The lung and OB were collected, homogenized, and analyzed for acridinium activity. C. Acridinium activity in a nasal wash of mice before and after suturing four nasolacrimal duct puncta 120 min after eyedrop administration with acridinium-labeled OVA plus CT. D. OVA-specific IgA Ab levels in serum and mucosal secretions of sutured and nonsutured mice following eyedrops with OVA (100 μg) plus CT (2 μg) or PBS alone every wk three times. *p < 0.05; **p < 0.01; ***p < 0.001 versus PBS-vaccinated group. Data represent one of three separate experiments, each with four mice per group. OB, olfactory bulb.

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 (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 × LD50; 500 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 × LD50; 1 × 106 PFU). We also compared the body weight changes between eyedrop-vaccinated and i.n.-vaccinated mice given live A/PR/8 virus (0.1 × LD50; 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
the RASV strain (14, 15). 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.

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

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).
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. 6B–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. 6C, 6D), when compared with PBS-treated mice (Fig. 6A, 6B). We found epithelial cells having depressed surfaces with irregular and short microvilli (Fig. 6D, arrow) and clusters of cells with more rounded tops and straighter microvilli, which were observed only in conjunctiva goblet cells (Fig. 6D, arrowhead) (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+ cells to internalize bacteria, we performed eyedrop administration using Salmonella-GFP and analyzed the results by vertical section. Of note, rSalmonella-GFP was located in the intracellular region of UEA-1+ cells on the conjunctiva (Fig. 8Be). Taken together, these results indicate that UEA-1+ cells in the nictitating membrane of conjunctiva have the ability to take up and internalize bacteria from the lumen and are involved in the induction of protective immunity via eye mucosa after eyedrop vaccination.

**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 immunities and 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 LNs during 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 suturing 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 present study, microvilli of 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 pigs 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 S. typhimurium enteritidis (7). Therefore, these cells in the conjunctiva might be considered as a strategy for mucosal vaccination.

In summary, this study shows that eye mucosa has a function as an inductive site for mucosal and systemic immunity. Although eyedrop vaccination might be considered as a strategy for mucosal vaccination, by the oral, i.n., and sublingual routes, eyedrop administration might be considered as a strategy for mucosal vaccination.


