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A Single Intraduodenal Administration of Human Adenovirus 40 Vaccine Effectively Prevents Anaphylactic Shock

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Vaccine administration into the intestine is known to induce mucosal tolerance most efficiently. Therefore, developing a delivery system that targets the intestinal mucosa is expected to improve the efficiency of immunosuppression. Human enteric adenovirus serotype 40 (Ad40)-based vectors have the advantage of targeting intestinal mucosa, making them prime candidates as mucosal vaccine carriers for immunosuppression. Here, after both oral and intraduodenal administrations, the vector distribution of replication-defective recombinant Ad40 vectors (rAd40) was significantly higher than that of a conventional Ad vector based on human adenovirus 5 (Ad5) in ilea containing Peyer's patches. Single intraduodenal administration of rAd40 induced antigen-specific mucosal immunoreaction mediated by intestinal mucosal and systemic immunity. In ovalbumin-induced allergy mouse models, this approach inhibited antigen-specific delayed-type hypersensitivity reactions, diarrhea occurrence, and systemic anaphylaxis. Thus, a single intraduodenal administration of rAd40 provides a potent method of inducing allergen-specific mucosal tolerance and a new allergen-specific immunotherapy for overcoming problems with current therapies against life-threatening allergic reactions, including anaphylaxis.

The prevalence of allergies has continued to rise in industrialized countries for more than 50 years. Sensitization rates to one or more common allergens among schoolchildren are approaching 40 to 50% (1). Allergen-specific immunotherapies, including recombinant hypoallergenic allergen-based vaccines, are the most promising procedures for overcoming problems with current therapies against allergies (2). Anaphylaxis is a life-threatening rapid allergic reaction, the lifetime prevalence of which is estimated at 0.05 to 2% based on international studies (3). At this time, approaches for preventing anaphylaxis are limited to strict avoidance of the specific trigger, immunomodulation, and protein-based subcutaneous vaccines with the relevant antigen.

Although almost all vaccines have been administered intramuscularly or subcutaneously, vaccine administration into the intestine induces mucosal immune responses most efficiently (4). In fact, the intestine is the largest immune organ in the body, containing the highest number of immune cells (5). Mucosal vaccination is known to induce substantial antibody responses in a wide variety of regions (e.g., small intestine, ascending colon, and mammary and salivary glands) (6). Nevertheless, sufficient stimulation of the intestinal immune response requires direct initiation of the intestinal mucosa (7). Particularly, Peyer's patches (PPs) are unique sites for the initiation of mucosal immunity and tolerance (8), where microfold cells within the follicle-associated epithelium of PPs transport antigens and microorganisms (9). Therefore, developing a mucosal delivery system that targets the intestinal mucosa, especially PPs, is expected to improve the efficiency of immunomodulation.

Vaccination using human adenovirus 5 (Ad5) has been tested in several clinical trials (10, 11). When Ad5 was evaluated by mucosal administration (e.g., oral gavage, intra-ileal, or intraduodenal administration using enteric-coated capsules) (12–14), the results did not show Ad5 to be a promising mucosal vaccine vector candidate, most likely due to its natural tropism for the respiratory tract. The presence of preexisting immunity to Ad5 has been reported to have an effect on the immunogenicity of Ad5 vectors in humans (15, 16). Levels of neutralizing antibodies to Ad40 are low

in children below 18 months old and adults above 70 years old (17). These data indicate that for babies, young infants, and older adults the Ad40 vector may be more suitable than the Ad5 vector. Differing from other adenoviruses, Ad40 contains two fibers. This special virion structure allows Ad40 to exhibit a unique mucosal tropism in the gastrointestinal tract (18). Thus, replication-defective recombinant Ad40-based vectors (rAd40) have an advantage for immunological interventions via intestinal mucosa binding, making them prime candidates for mucosal vaccine carriers.

We hypothesized that recombinant Ad40 vector (rAd40) vaccines might induce mucosal tolerance against allergy and anaphylaxis mediated by both intestinal mucosal and systemic immunity. Although Ad40 is called the fastidious virus, as it is extremely difficult to culture *in vitro* (19, 20), we recently established a novel vector generation method for rAd40, as previously described (21, 22).

Here, we report a novel rAd40 vaccine, not only for inducing allergen-specific intestinal mucosal tolerance but also for controlling severe allergic reactions and anaphylaxis.

MATERIALS AND METHODS

Mice. All animal experiments were conducted with approval from the Institutional Animal Care and Use Committee, University of Minnesota. In all cases, 6- to 7-week-old female C57BL/6 (B6) or BALB/c mice (Harlan Laboratories, Inc., Madison, WI) were used.

Oral administration. We used a partially E1-deleted rAd40 (Ad40pΔE1) with a luciferase reporter gene (Luc) cassette and cytomegalovirus (CMV)-promoter-driven Luc or ovalbumin (Ova) cassette (rAd40pΔE1-Luc, rAd40pΔE1-CMV-Luc, or rAd40pΔE1-CMV-Ova) for our experiments, as we previously reported (22). The B6 mice (6 mice per group) were anesthetized with 2 to 5% isoflurane after access to food

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was prohibited for 4 h to limit foods in the stomach. Oral gavage was performed using a ball-ended feeding needle (FTP-20-30, Instech Solomon, Plymouth Meeting, PA). The distance that the needle had to be inserted into the mice was marked on the needle. The anesthetized mice were restrained in a position with the head and body extended as straight as possible, and the gavage needle was introduced into the space between the left incisors and molars, and swallowing was gently induced as the feeding tube approached the pharynx, facilitating entry into the esophagus. Once the desired position was attained, 100 μ l phosphate-buffered saline (PBS) (control) or 10^{10} virus particles (VP) of rAd diluted in PBS were administered and the needle was withdrawn. To ensure that there were no adverse effects, the mice were monitored every day after the procedure until 48 h after vector administration.

Intraduodenal administration. Following 4 h of fasting for reducing the food in the duodenum, intraduodenal (i.d.) administration was accomplished by aseptically opening the abdomens of B6 (for *in vivo* biodistribution and delayed-type hypersensitivity) or BALB/c (for diarrhea and anaphylaxis models) mice anesthetized with isoflurane followed by injecting 100 μ l PBS (control) or 10^{10} VP of rAd in PBS with a 29-gauge 1/2-inch needle into the lumen of the duodenum (1 cm downstream of the stomach). The abdominal wall and skin were closed by suture.

In vivo biodistribution. Forty-eight hours after oral or i.d. administration, B6 mouse duodenum (only for oral administration), jejunum without PP (2-cm median part of the small intestine without macroscopically visible PP), ileum without PP (2 cm upstream of the cecum without visible PP), ileum containing PPs (visible PPs observed immediately upstream of the cecum), colon (2 cm downstream of the cecum), mesenteric lymph nodes (MLNs), spleen, and liver were isolated, and DNA was purified by a QIAamp DNA blood minikit (Qiagen, Valencia, CA). In order to compare the DNA copy numbers between rAd40p Δ E1-CMV-Luc and rAd5-CMV-Luc, previously published primers for the luciferase reporter gene and mouse GAPDH were used in real-time PCR analysis with the SYBR green method. The real-time PCR assay was performed using a QuantiTect SYBR green PCR kit (Qiagen) with an ABI sequence detection system (ABI Prism 7400, Applied Biosystems), and each experiment was conducted for at least three independent experiments, each performed in triplicates. PCR-cloned Ad40 fiber, luciferase reporter, and mouse GAPDH fragments were used as copy number standards. Template-negative samples served as controls for real-time PCR and were always subdetectable. Tissues were analyzed by hematoxylin and eosin (H&E) staining and luciferase staining.

Immunohistochemistry. Luciferase proteins in B6 mouse tissues after oral and i.d. administrations were detected by immunohistochemistry with a 1:100 dilution of a primary goat anti-luciferase (Promega, Madison, WI) antibody and a 1:200 dilution of secondary polyclonal rabbit anti-goat IgG-horseradish peroxidase (HRP) (Dako, Glostrup, Denmark). Tissues were fixed with 4% paraformaldehyde (PFA) in PBS, embedded in paraffin wax, sectioned at 3 μ m, and stained sequentially with H&E staining as well as immunohistochemistry for luciferase proteins. Immunohistochemistry was performed with an EnVision+ system-HRP (diaminobenzidine [DAB]) (Dako). Formalin-fixed paraffin-embedded tissue sections were sequentially deparaffinized, rehydrated, boiled in 0.1 M citrate buffer (pH 6.0; Dako) for antigen retrieval, and blocked for nonspecific activity with 1% bovine serum albumin (Sigma-Aldrich) and protein block serum-free (Dako). Incubation with primary antibody was performed overnight at 4°C. After a brief rinse in PBS with 0.1% Tween 20, the sections were incubated with secondary antibody for 30 min and with DAB+ substrate-chromogen solution (Dako) at room temperature. The slides were mounted in Glycergel mounting medium (Dako). The histological findings of mouse tissues were assessed with H&E staining of slides. All stainings were performed in at least three independent experiments. All slides were scanned at $\times 100$, $\times 200$, and $\times 400$ magnification using a Nikon Eclipse TS100 microscope (Nikon, Melville, NY). The primary antibody was omitted on the slides for negative controls, where the signals were not detected.

Immunofluorescence staining. The frozen B6 mouse tissues for H&E stains were fixed with 4% PFA at room temperature for 10 min. Frozen sections (6 to 10 μ m) were cut on a cryostat and prepared for staining experiments. After the evaluation of tissues using H&E stain, the sections were fixed with cold acetone (-20°C) for 10 min. Tissues were then rinsed with PBS, blocked with 1% BSA in 0.1% Tween 20–PBS, incubated with the primary monoclonal antibody (Foxp3, 1:50; eBioscience, San Diego, CA) overnight, and followed by incubation with a tetramethyl rhodamine isothiocyanate-conjugated secondary antibody (Jackson Immuno Research Labs, West Grove, PA) for 1 h, and fluorescein isothiocyanate (FITC)-anti-mouse CD4 antibody (eBioscience) or FITC isotype control (for negative control) for 1 h. Finally, DAPI (4',6'-diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA) was used as a nuclear counterstain. Images were acquired using a confocal spectral imaging microscope system (Nikon). Only the unstained and secondary antibody control sections were used to determine the levels of background tissue autofluorescence and to identify any potential nonspecific binding of the secondary antibodies. For negative controls, the tissues from naive mice were used in every staining. Each stain was carried out on at least three individual mice per group, and the images in the figures are representative of each group. The numbers of Foxp3 $^{+}$ and CD4 $^{+}$ cells were counted in >10 high-power fields (HPF) ($\times 400$) per tissue ($n = 6$ in each group, in triplicate).

Isolation of lymphocytes and flow cytometry. The isolated MLNs were incubated in RPMI 1640 (Mediatech, Inc., San Jose, CA) with 100 U/ml collagenase D and 10 U/ml RNase-free DNase for 30 min, agitated with scissors or scalpels, and then passed through 70- and 40- μ m strainers. Splenocytes were prepared by passing through a 100- μ m strainer. Erythrocytes were removed using red blood cell lysis buffer (BioLegend, San Diego, CA) for 5 min followed by washes in PBS and passing through a 40- μ m strainer. Mononuclear cells were prepared in lymphocyte separation medium (Mediatech, Inc.) and passed through 40- μ m strainers. Viable lymphocytes were counted using a hemacytometer and trypan blue.

Lymphocytes from MLNs and splenocytes at 14 or 42 days after i.d. administration with rAd (i.d. rAd) were harvested from B6 mice. For analysis of delayed-type hypersensitivity responses, splenocytes were isolated at 7 days after subcutaneous (s.c.) Ova challenge. Lymphocytes ($>5 \times 10^5$) or splenocytes ($>1 \times 10^6$) were directly analyzed by flow cytometry or placed in culture, depending on the analyses. The regulatory T cells were identified as CD4 $^{+}$ CD25 $^{+}$ Foxp3 $^{+}$ cells using an eBioscience Foxp3 staining buffer set (eBioscience). Prior to intracellular cytokine staining, splenocytes (2×10^6 /ml) were incubated with 10 μ g/ml of Ova peptide (SIINFEKL, H-2K ^{b} ; ProImmune), Pan02-Ova cell lysate (generated by four freeze-thaw cycles, passed through a 0.22- μ m filter, and total protein concentration as determined by a DC protein assay), or 0.1% dimethyl sulfoxide (DMSO) and PBS (control) in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 IU/ml penicillin and 100 μ g/ml streptomycin for 6 h. BD GolgiPlug (BD Biosciences, San Jose, CA) was added for the last 4 h of *ex vivo* culture. After surface staining with anti-CD3e, -CD4, -CD8a, or -NK1.1 (BD Biosciences), cells were stained with anti-gamma interferon (IFN- γ), anti-interleukin 4 (IL-4), and anti-IL-10 using a Cytofix/Cytoperm kit (BD Biosciences). Over 2×10^5 events were acquired on a FACSCalibur or LSRII (Becton, Dickinson, Franklin Lakes, NJ). Data were analyzed using CellQuest (Becton, Dickinson) and FlowJo (TreeStar, San Carlos, CA) software. Cells in the lymphocyte gate were used for analysis. As a negative control, cells were stained with isotype controls.

Evaluation of Ova-specific IgG1, IgG2a, and IgE levels in serum or plasma, and IgA in feces. Blood samples were drawn from the retro-orbital plexus using heparin-coated or noncoated glass capillaries or obtained by cardiac puncture in heparin-coated or noncoated syringes immediately following sacrifice, and allowed to clot at 37°C for 2 h (for serum). The clots from serum or cells from plasma were removed by

centrifuging for 15 min at 10,000 rpm at 4°C, and the supernatant was stored at -80°C.

Fecal pellet samples were collected and extracted by making a 1/10 suspension (wt/vol) with PBS plus protease inhibitor. After the suspension, samples were vortexed and spun for 10 min at 14,000 rpm. The supernatant was stored at -80°C.

Sera and feces were tested at 1/10 and 1/50 (for feces) or 1/100 (for sera or plasma) in duplicate. Serum or plasma IgG1, IgG2a, and IgE and fecal IgA concentrations were calculated with controls placed in each enzyme-linked immunosorbent assay (ELISA) plate (mouse anti-ovalbumin IgG1, IgG2a, IgE, or IgA ELISA kit; Alpha Diagnostic International, San Antonio, TX). The positive controls gave the optical density readouts (>0.3 for sera or plasma, >0.5 for feces). Results are presented as activity units according to the user manual.

Induction and measurement of delayed-type hypersensitivity. The effects of intraduodenally administered control (PBS), and 10^{10} VP of rAd40p Δ E1-CMV-Ova and rAd5 Δ E1-CMV-Ova were compared at 14 days after i.d. rAd to B6 mice for delayed-type hypersensitivity assay, referred to in a previous report (23). Four weeks after i.d. rAd, B6 mice were immunized with an s.c. administration of 200 μ l PBS/complete Freund's adjuvant emulsion containing 300 μ g Ova protein (A-5503; Sigma-Aldrich) into the tail base. Fourteen days after immunization, the mice were challenged by s.c. administration of 50 μ g Ova in 20 μ l PBS into the right ear pinna, while 20 μ l PBS without Ova was injected into the left ear pinna as a control. The swelling was measured with a micrometer in a blinded fashion before s.c. administration and 48 or 72 h after s.c. administration. Ova-specific swelling was calculated as follows: (right thickness - left thickness)_{48 or 72 h} - (right thickness - left thickness)_{0 h}. Serum and spleen were extracted from sacrificed mice for ELISA and flow cytometry to assess T cell responses 7 days after s.c. Ova challenge into the ear pinna.

Diarrhea model. Diarrhea and other allergic parameters were compared among control (PBS) mice and BALB/c mice pretreated with 10^{10} VP of rAd40p Δ E1-CMV-Ova- and rAd5 Δ E1-CMV-Ova at 14 days after i.d. rAd in allergic diarrhea models, based on a previous report (24). Four weeks after i.d. rAd, BALB/c mice were sensitized twice, 2 weeks apart, by intraperitoneal administration of 50 μ g Ova in the presence of 1 mg of aluminum potassium sulfate adjuvant (A-7210; Sigma-Aldrich). Two weeks later, the mice started to receive i.g. administration of 50 mg Ova protein in 250 μ l PBS every 2 days, which was continued until there were two observations of diarrhea in 100% of the control mice. Before each i.g. Ova challenge, mice were deprived of food for 4 h with the aim of limiting antigen degradation in the stomach. Diarrhea was assessed by visually monitoring mice for up to 1 h following i.g. challenge. Mice demonstrating liquid stool were recorded as diarrhea-positive animals. At the second occurrence of diarrhea in every control mouse, plasma and feces were extracted for ELISA at 1 h after the final intragastric (i.g.) Ova challenge.

Systemic anaphylaxis, intestinal vascular permeability, and eosinophil counts. Forty-eight hours after final i.g. challenge in allergic diarrhea models, BALB/c mice were administered Ova (100 μ g/mice, for systemic anaphylaxis) in Evan's blue dye (EBD) (20 mg/kg, for intestinal vascular permeability assay) in PBS (200 μ l) intravenously (i.v.) by tail vein injection. The anaphylactic reactions were monitored at 5, 10, 15, 30, 45, and 60 min after i.g. Ova challenge. Fifteen minutes after i.v. Ova challenge, blood was drawn from the tail vein for analyzing plasma histamine level. Vascular permeability was assessed by Evan's blue dye extravasation as previously described (25, 26). One hour after intravenous (i.v.) challenge, mice were sacrificed, and cardiac puncture was performed for peripheral blood collection into EDTA-containing tubes, and heart perfusion was performed with 10 ml PBS. The jejunum (10-cm median part of the small intestine), ileum (10 cm upstream of the cecum), and colon (10 cm downstream of the cecum) were harvested and allowed to dry for 24 h at 37°C, and dry weight was determined. Evan's blue dye (EBD) was extracted by homogenization in formamide (20 ml/g dry tissue) (Sigma-Aldrich), and incubated for 24 h at 50°C. Homogenized samples were centrifuged at 14,000 rpm for 30 min, and the supernatants were measured at 620 nm in

a spectrophotometer. The extravasated EBD concentrations were calculated against a standard curve, and the data were expressed as micrograms of EBD per gram of dry tissue weight. Eosinophil counts in bone marrow cells were performed using a method based on a previous report (27). For counting eosinophils in bone marrow cells, slides were stained with May-Grunwald-Giemsa, differential cell counts were performed under a light microscope, and the total number of 10 microscopic high-power fields ($\times 400$, >100 cells total) provided the final counts.

Statistical analysis. Data are presented as mean values of at least three independent experiments, and error bars indicate the 95% confidence interval. Continuous variables were compared by two-way analyses of variance (ANOVA). All *P* values were 2 sided, and differences were considered statistically significant at *P* values of <0.05. In all figures, the asterisks denote statistically significant differences when comparing the indicated groups.

RESULTS

Biodistribution of Ad vectors expressing luciferase *in vivo*. The biodistributions of rAds at 48 h after oral (Fig. 1A) and i.d. (Fig. 1B) administrations in B6 mice (shown as relative virus copy numbers of Luc DNA per mouse GAPDH DNA) were analyzed by real-time PCR. The vector distribution of rAd40p Δ E1-CMV-Luc (rAd40-Luc) was significantly higher than that of rAd5-CMV-Luc (rAd5-Luc) in the ileum containing PPs and MLNs. Furthermore, significant levels of rAd5-Luc were detectable in the jejunum (without PP). With oral administration, the distribution of rAd5-Luc was significantly higher than rAd40-Luc in the ileum without PP, and the distribution of rAd40-Luc was significantly higher than rAd5-Luc in the colon. Conversely, after i.d. rAd, the distribution of rAd40-Luc was significantly higher than rAd5-Luc in the ileum without PP and spleen, and the distribution of rAd5-Luc was significantly higher than rAd40-Luc in the colon. Thus, rAd40 showed a significantly higher vector distribution in PPs and MLNs compared to conventional rAd5.

Figure 1C shows *in vivo* biodistribution evaluated by luciferase immunohistochemistry in the ileum containing PPs of B6 mice at 48 h after oral and i.d. administrations. After oral and i.d. administrations of rAd40p Δ E1-CMV-Luc, strong luciferase expression was localized in the ileum around the PPs' domes. However, scarce luciferase expression was found in the jejunum and ileum after oral and i.d. administrations of rAd5-CMV-Luc. Luciferase expression was not detectable in MLNs or spleen after i.d. administration with rAd40p Δ E1-CMV-Luc and rAd5-CMV-Luc.

Next, *in vivo* expression of transgene via rAd was evaluated by the presence of luciferase immunofluorescence at 48 h after i.d. administrations. Consistent luciferase expression was observed in the ileum containing PPs of B6 mice after rAd40-Luc i.d., while expression was found in the jejunum after rAd5-Luc i.d. (Table 1). Luciferase expression was not detected in the MLNs, spleen, or liver after i.d. rAd40-Luc and rAd5-Luc. These data demonstrate that higher distributions of transgenes expressed by rAd40 can be observed in the ileum containing PPs after both oral and i.d. administration.

Immune responses of Ad vectors expressing ovalbumin *in vivo*. The change of *in vivo* immune response induced by the specific antigen-expressing rAd was determined with Ova- and Luc (for negative control)-expressing rAd, because Ova is widely used as a model antigen. The proportions of Foxp3⁺ of CD4⁺ cells in MLNs were increased by i.d. rAd in B6 mice compared to control mice at day 14 (Fig. 2A). The i.d. rAd40p Δ E1-CMV-Ova (rAd40-

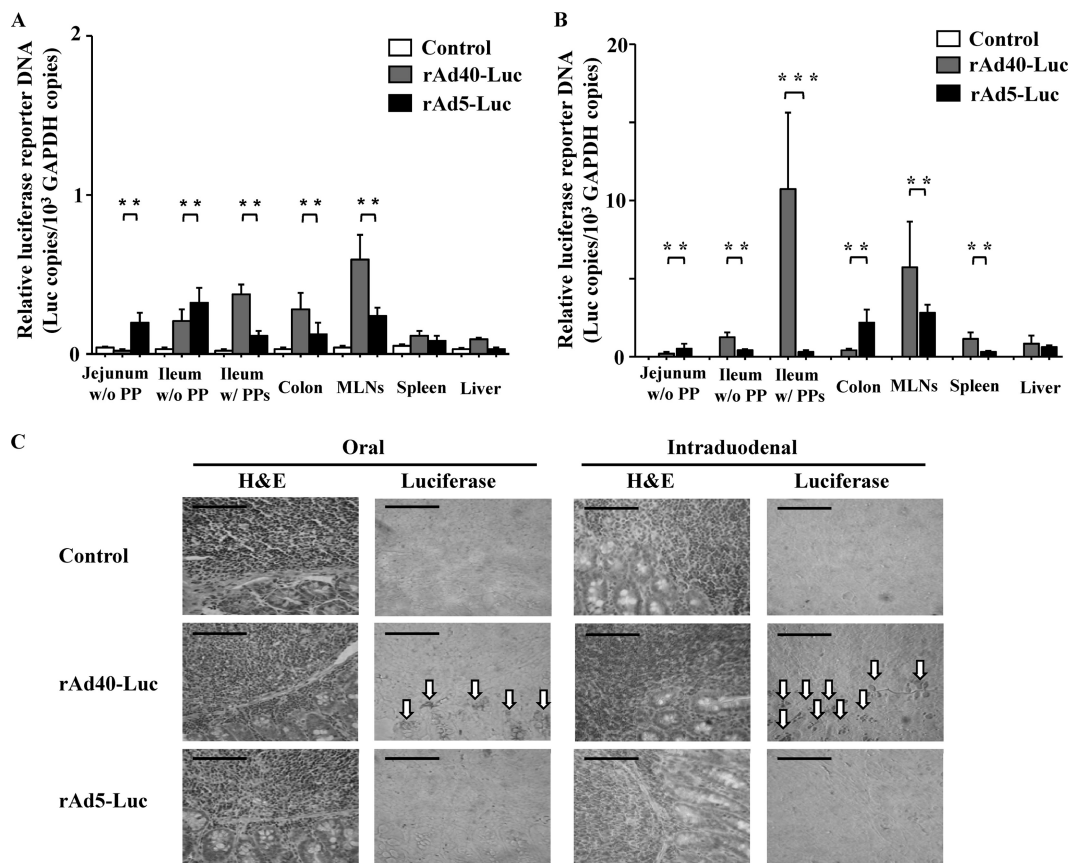


FIG 1 *In vivo* biodistribution of Ad vectors expressing luciferase. (A, B) *In vivo* distribution of rAd40 and rAd5 expressing Luc following oral and intraduodenal (i.d.) administrations in B6 mice. (A) The relative copy numbers of Luc (Luc DNA per mouse GAPDH DNA) at 48 h after oral administration. (B) The luciferase copy number at 48 h after i.d. administration with rAd (i.d. rAd). PPs, Peyer's patches; MLNs, mesenteric lymph nodes; w/o, without; w/, with (containing); C, control; 40, rAd40pΔE1-CMV-Luc; 5, rAd5-CMV-Luc. (C) Detection of transgene expression in mouse ileum around Peyer's patches' domes following oral and intraduodenal administrations with rAd40 and rAd5 expressing luciferase. Luciferase expression was evaluated by immunohistochemistry in the ileum of B6 mice at 48 h after oral and i.d. administrations of rAd40pΔE1-CMV-Luc and rAd5-CMV-Luc. After oral and i.d. administrations of rAd40pΔE1-CMV-Luc, strong luciferase expression was localized in the ileum around PPs' domes (white arrows). Conversely, expression in the region was not shown after administrations of rAd5-CMV-Luc. Bar = 100 μm. H&E, hematoxylin and eosin staining; Ad40, rAd40pΔE1-CMV-Luc; Ad5, rAd5-CMV-Luc. Each group included 3 to 6 independent experiments in triplicate. **, $P < 0.01$; ***, $P < 0.001$.

Ova) induced the highest proportion of Foxp3⁺ compared to CD4⁺ cells in MLNs, while i.d. rAd5-CMV-Ova (rAd5-Ova) induced significantly lower proportions of Foxp3⁺ cells. The proportions of regulatory T (Treg) CD4⁺ CD25⁺ Foxp3⁺ to CD4⁺ cells in MLNs at 42 days post i.d. rAd were maintained at higher

levels compared to those in control mice. Again, i.d. rAd40-Ova induced the highest numbers of Tregs in MLNs, while i.d. rAd5-Ova induced significantly lower numbers (Fig. 2B). These results showed that rAd40-Ova can induce intestinal mucosal immunity to a greater extent than rAd5-Ova.

To further characterize the systemic immune response, we measured levels of Ova-specific IgG1, IgG2a, and IgE in serum as well as in fecal IgA as markers of the T-helper (Th) immune response. Ova-specific IgG1 was detectable in serum by rAd40-Ova and rAd5-Ova at 14 days after i.d. rAd but not detected after oral administration of rAd (data not shown). The peaks of the IgG1 activity units were measured at 14 days in rAd5-Ova and 21 days in rAd40-Ova (Fig. 2C). IgG1 activity units in rAd40-Ova were significantly higher than those in rAd5-Ova at 21, 28, and 35 days post i.d. rAd. Serum IgG2a and IgE and fecal IgA were not detectable between days 0 and 42. While rAd40 and rAd5 induced systemic immunity along a Th2 cell-dependent pathway, rAd40 induced a more robust systemic immune response with a later peak than rAd5.

Inhibition of ovalbumin-induced delayed-type hypersensitivity response. To evaluate whether it was possible to induce

TABLE 1 *In vivo* luciferase immunohistochemical detection^b

Sample type	No. of mice with transgene expression/total no. of mice for:		
	Control	rAd40-Luc	rAd5-Luc
Jejunum without PPs	0/9 ^a	2/9	9/9
Ileum without PPs	0/9	2/9	2/9
Ileum with PPs	0/9	9/9	0/9
Colon	0/9	3/9	4/9
MLNs	0/9	0/9	0/9
Spleen	0/9	0/9	0/9
Liver	0/9	0/9	0/9

^a Three different samples from three different sites in each mouse are shown.

^b PPs, Peyer's patches; MLNs, mesenteric lymph nodes; rAd40-Luc, rAd40pΔE1-CMV-Luc; rAd5-Luc, rAd5-CMV-Luc.

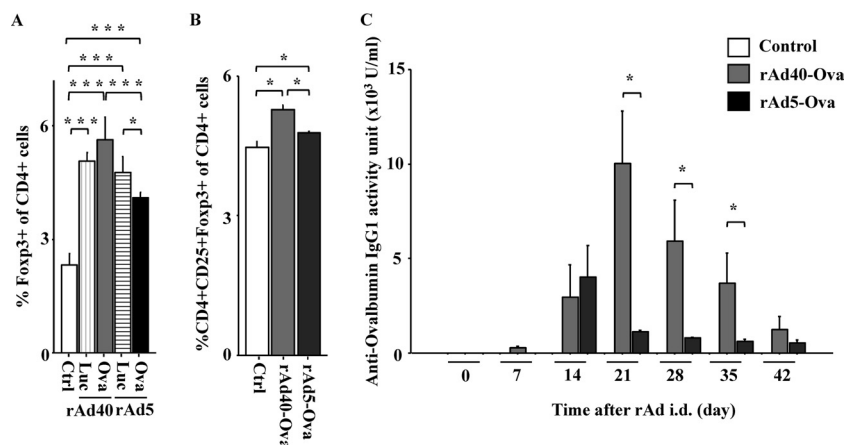


FIG 2 Mucosal and systemic immune responses induced by a single intraduodenal administration of Ad vectors in B6 mice. (A) The number of Foxp3⁺ cells in MLNs at 14 days after i.d. administration with control (Ctrl), rAd40pΔE1-CMV-Luc (rAd40-Luc), rAd40pΔE1-CMV-Ova (rAd40-Ova), rAd5-CMV-Luc (rAd5-Luc), or rAd5-CMV-Ova (rAd5-Ova). (B) The numbers of CD4⁺ CD25⁺ Foxp3⁺ Tregs in MLNs at 42 days after i.d. administration with control, rAd40-Ova, or rAd5-Ova. (C) The activities of Ova-specific IgG1 in serum at 0, 7, 14, 21, 28, 35, and 42 days after i.d. administration with control, rAd40-Ova, or rAd5-Ova. *, $P < 0.05$; ***, $P < 0.001$.

systemic tolerance after i.d. rAd-Ova in B6 mice, delayed-type hypersensitivity (DTH) reactions were tested (Fig. 3A). Both 48 and 72 h after subcutaneous (s.c.) Ova challenge, DTH reactions were reduced only by rAd40-Ova (Fig. 3B). The proportions of splenic Ova-specific IFN- γ -secreting CD8⁺ T cells to CD8⁺ T cells and IFN- γ -secreting CD4⁺ T (IFN- γ ⁺ CD3e⁺ CD4⁺) cells to CD4⁺ T cells were decreased by rAd40-Ova at 7 days after s.c.

challenge (Fig. 3C). These data suggest that Ova-specific activation of lymphocytes in the systemic immunity was suppressed by i.d. rAd40-Ova.

Inhibition of ovalbumin-induced diarrhea. To examine the inhibition of Ova-induced diarrhea in rAd40-Ova-pretreated BALB/c mice, we used a food allergy model (Fig. 4A). We observed the percentage of diarrhea occurrence following each of the 9 in-

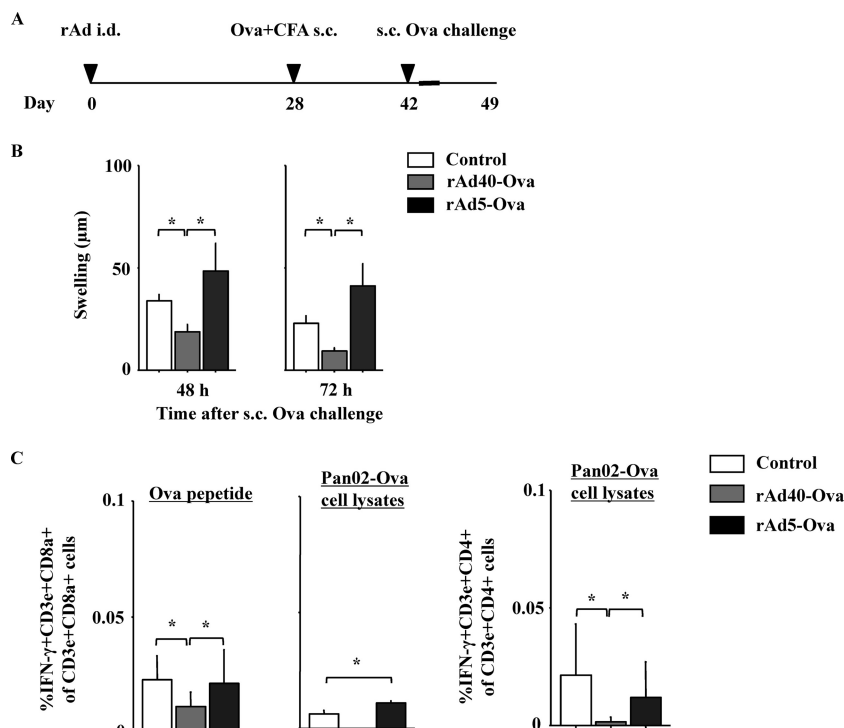


FIG 3 Delayed-type hypersensitivity responses. (A) Timeline illustrating B6 mice immunized with a subcutaneous (s.c.) administration of Ova plus complete Freund's adjuvant (CFA) at 28 days after i.d. administration with control, rAd40-Ova, or rAd5-Ova. Fourteen days after Ova immunization (42 days after i.d. rAd), an ear pinna was challenged with Ova s.c. and swelling was measured at 48 and 72 h after s.c. Ova challenge. (B) The diameters of the ear pinna swelling in control and rAd40-Ova- or rAd5-Ova-pretreated mice measured at 48 and 72 h after s.c. challenge. (C) Seven days after s.c. Ova challenge (49 days after i.d. rAd), spleen was harvested to assess IFN- γ -secreting Ova-specific CD8⁺ or CD4⁺ T cell reactions and analyzed by flow cytometry. *, $P < 0.05$.

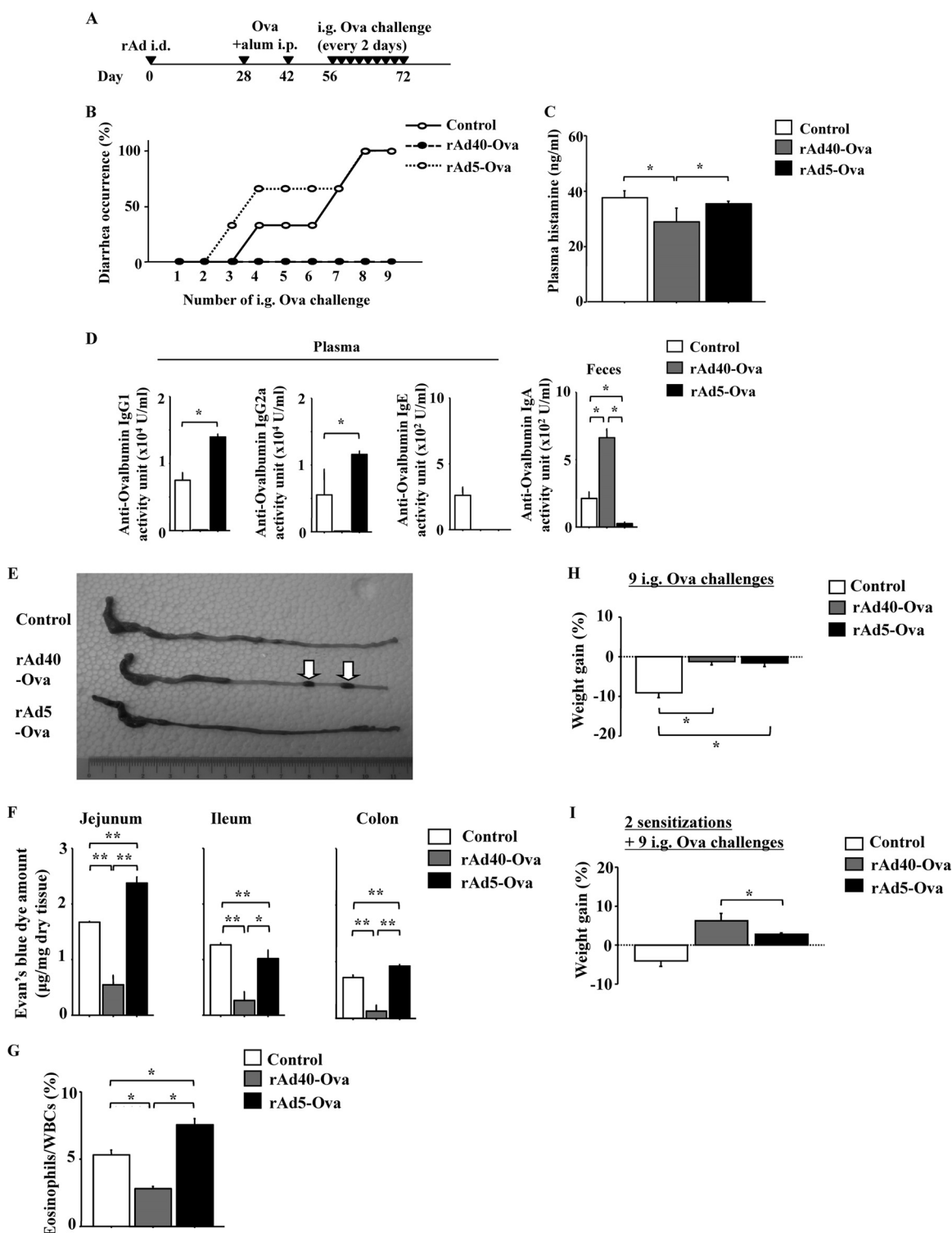


FIG 4 Mouse model of ovalbumin-induced diarrhea. (A) BALB/c mice were sensitized by an intraperitoneal (i.p.) administration of Ova plus aluminum potassium sulfate adjuvant (alum) at 28 and 42 days after i.d. administration with control, rAd40-Ova, or rAd5-Ova and then challenged with i.g. administration of Ova every 2 days at 56 days after i.d. rAd. (B) Diarrhea occurrence assessed for 1 h after i.g. Ova challenge in control and rAd40-Ova- or rAd5-Ova-pretreated mice. (C) Plasma histamine levels after 1 h following the ninth i.g. challenge. (D) Ova-specific plasma IgG1, IgG2a, and IgE and fecal IgA after 1 h following the ninth i.g. challenge. (E) Macroscopic view of cecum and colon 60 min after i.v. challenge. White arrows indicate solid stool pellets. (F, G) Intestinal vascular permeabilities (F) and eosinophil counts of bone marrow (G) 60 min after i.v. challenge. WBCs, white blood cells. (H, I) Weight change after ovalbumin-induced diarrhea. The percentage change of body weight from (H) pre-i.g. challenge (56 days after rAd i.d.) or (I) presensitization (28 days after rAd i.d.) to the end of the study (72 days after rAd i.d.). *, $P < 0.05$; **, $P < 0.01$.

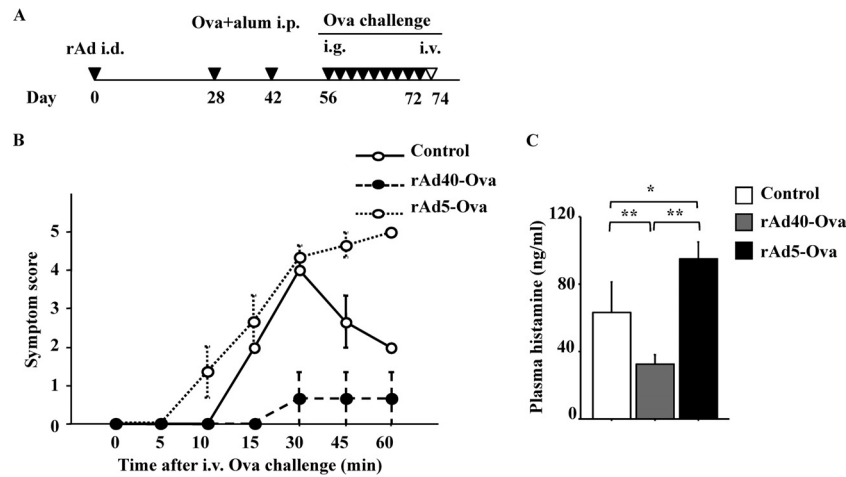


FIG 5 Systemic anaphylaxis models. (A) BALB/c mice were sensitized by an i.p. administration of Ova plus alum at 28 and 42 days after i.d. administration with control, rAd40-Ova, or rAd5-Ova and then challenged with the ninth i.g. administration of Ova at 56 days after i.d. rAd and an i.v. Ova challenge at 74 days. (B) Symptom score in control and rAd40-Ova- or rAd5-Ova-pretreated mice. (C) Plasma histamine levels 15 min after i.v. challenge. *, $P < 0.05$; **, $P < 0.01$.

tragastric (i.g.) Ova challenges (Fig. 4B). After 8 consecutive i.g. Ova challenges, profuse liquid stool was observed in all control mice, while small amounts of liquid stool and frequent bowel movements were observed in all rAd5-Ova-pretreated mice. No diarrhea was observed in any rAd40-Ova-pretreated mice. One hour following the last i.g. challenge, plasma histamine levels (Fig. 4C) and the activities of plasma Ova-specific IgG1 and IgG2a (Fig. 4D) were significantly suppressed in rAd40-Ova-pretreated mice. However, the activities of fecal Ova-specific IgA were significantly increased in rAd40-Ova-pretreated mice compared to control and rAd5-Ova-pretreated mice, corresponding to the inhibition of Ova-induced diarrhea occurrence with rAd40-Ova-pretreated mice. Plasma Ova-specific IgE, a mediator for type I hypersensitivity, was mobilized only in control mice.

After 9 i.g. challenges and 60 min after i.v. challenge, diarrhea was noted by macroscopic observation of the colon and cecum, illustrated by the liquid stool observed in all control and rAd5-Ova-pretreated mice. On the contrary, solid stool pellets were found in the distal colon of all rAd40-Ova-pretreated mice (Fig. 4E). The rAd5-Ova-pretreated mice exhibited markedly red colons after anaphylactic shock, and bloody diarrhea was observed in the jejunum.

To evaluate vascular permeability, Evan’s blue extravasation in the tissues was measured. Evan’s blue concentrations throughout the intestinal system (jejunum, ileum, and colon) of rAd40-Ova-pretreated mice were significantly lower than those in control and rAd5-Ova-pretreated mice (Fig. 4F). The jejuna of rAd5-Ova-pretreated mice showed significantly more Evan’s blue extravasation than those of control mice, coinciding with the observation of bloody diarrhea and the detection of higher luciferase copy numbers or constant luciferase expression of rAd5-Luc in the *in vivo* biodistribution assay. Greater extravasation was observed in the colon of rAd5-Ova-pretreated mice compared to control mice, corresponding with remarkable redness.

To evaluate the systemic responses, numbers of bone marrow eosinophils were measured at 60 min after i.v. challenge (Fig. 4G). The number of eosinophils was lower in rAd40-OVA-pretreated mice, reflecting plasma histamine levels at 15 min.

As a marker specific to our diarrhea model, body weight was

monitored every 3 days. During both i.g. challenges (from the first challenge to 1 h following the last [ninth] challenge) and the period of Ova sensitization and i.g. challenge, control mice showed significant body weight loss compared to rAd40-Ova- and rAd5-Ova-pretreated mice (Fig. 4H, I). During the time of sensitization and i.g. challenge, the level of weight gain in rAd5-Ova-pretreated mice was significantly lower than that in rAd40-Ova-pretreated mice. Thus, the change of body weight was different between groups, corresponding to diarrhea occurrence.

Inhibition of ovalbumin-induced systemic anaphylaxis. The effect of pretreatment on severe systemic allergic reaction was analyzed in an anaphylaxis model (Fig. 5A). Systemic anaphylaxis was measured by a symptom score (Table 2) (28) up to 60 min after an intravenous (i.v.) Ova challenge to Ova-induced diarrhea models (48 h after final i.g. challenge following allergic diarrhea models) (Fig. 5B). All control mice developed behavioral symptoms of “no activity” (score 4) at 30 min after i.v. challenge but recovered to score 2 (less severe) within 60 min. All rAd5-Ova-pretreated mice progressed to score 5 (death) due to anaphylactic shock within 60 min. In contrast, fewer and less severe (score 0 to 2) responses were seen in rAd40-Ova-pretreated mice after i.v. challenge. After 9 i.g. challenges and 15 min after i.v. challenge, plasma histamine levels in rAd40-Ova-pretreated mice were similar to levels after 1 h following the last i.g. challenge (before i.v. challenge), but levels in control and rAd5-Ova-pretreated mice exhibited a remarkable increase and levels in rAd5-Ova-pretreated mice were higher than in control mice (Fig. 5C). These

TABLE 2 Anaphylactic symptom score

Score	Symptom(s)
0	No sign of reaction
1	Scratching and rubbing around nose and head
2	Decreased activity with an increasing respiratory rate, pillar erecti, and/or puffing around the eyes
3	Labored respiration and cyanosis around the mouth and tail
4	Slight or no activity after prodding or tremors and convulsion
5	Death

results indicated that rAd40-Ova inhibited systemic anaphylactic reactions, while rAd5-Ova pretreatment resulted in allergic reactions worse than those in control mice.

DISCUSSION

Our data showed that when administered into the intestinal tract, rAd40 is more suitable than rAd5 as a vector for targeting intestinal mucosa. A single dose of intraduodenally administered rAd40 induced intestinal and systemic tolerance and inhibited DTH, diarrhea occurrence, and systemic anaphylaxis against Ova.

In PPs in mouse ileum, rAd40 showed higher luciferase reporter DNA copy numbers and luciferase expression than rAd5. Ad40, like Ad41 (29), lacks the RGD motif in the penton base (30). The natural tropism of Ad5 binding to the epithelial cells in the gastrointestinal tract requires not only coxsackievirus and adenovirus receptor binding but also integrin binding by RGD motifs in the penton base, similar to Ad5 binding to the liver (31). These reports suggest that rAd5 can bind to a wide variety of epithelial cells in the gastrointestinal tract, which may also explain why rAd5 did not show the gene expression around PPs after oral and i.d. administrations.

Different types of mucosal and systemic immune responses were induced by rAd40-Ova compared with rAd5-Ova, which may be explained by differences of *in vivo* biodistribution (i.e., ileum containing PPs for rAd40 and jejunum without PPs for rAd5). The numbers of Tregs in MLNs at 14 and 42 days after rAd40-Ova i.d. were higher than those of rAd5-Ova. These observations illustrate that rAd40 i.d. may induce intestinal tolerance mediated by intestinal immune cells.

Levels of mucosal and systemic tolerance were effectively induced with rAd40-Ova. DTH to Ova was remarkably reduced by rAd40-Ova pretreatment. Moreover, rAd40-Ova completely suppressed diarrhea occurrence and systemic anaphylaxis, corresponding with histamine levels, along with the activities of Ova-specific IgG1, IgG2a, and IgE; intestinal vascular permeability; and the number of bone marrow eosinophils. While there were symptoms of systemic anaphylaxis in all control mice recovered within 60 min after i.v. Ova challenge, all rAd5-Ova-treated mice died. rAd5-Ova worsened DTH, type IV hypersensitivity mediated by T cells, and the cross-reaction of both type I and IV hypersensitivity-induced lethal responses in mice. These *in vivo* results indicate that rAd40-Ova treatment of mice can inhibit both type I and IV hypersensitivity.

In this study, rAd40 i.d. showed promising preventive properties against Ova in mice. It added important advantages to Ad-based gene therapies, including intestinal mucosal availability, stimulation of mucosal and systemic immunity, and induction of allergen-specific intestinal mucosal tolerance. Additionally, our methods demonstrate the availability of recombinant allergens against anaphylaxis. The use of mouse models has led to many advances in the development of novel vaccines. In fact, work on mice resulted in successful vaccinations for the treatments of cancer. However, mice are not always reliable as preclinical models for human diseases, and the scientific literature is littered with examples of drugs that worked well in animals but turned out to be ineffective in clinical trials on humans. Therefore, we need to investigate rAd40 vaccination further. However, it may be useful not only for patients with food allergies, especially to eggs, but also for preventive properties against chronic local allergies such as hay fever, asthma, or autoimmune diseases.

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REFERENCES

1. Pawankar R, Canonica GW, Holgate ST, Lockey RF. 2011. WAO white book on allergy 2011–2012: executive summary. World Allergy Organization, Milwaukee, WI.
2. Valenta R, Linhart B, Swoboda I, Niederberger V. 2011. Recombinant allergens for allergen-specific immunotherapy: 10 years anniversary of immunotherapy with recombinant allergens. *Allergy* 66:775–783.
3. Simons FE, Arduoso LR, Bilò MB, El-Gamal YM, Ledford DK, Ring J, Sanchez-Borges M, Senna GE, Sheikh A, Thong BY, World Allergy Organization. 2011. World Allergy Organization anaphylaxis guidelines: summary. *J. Allergy Clin. Immunol.* 127:587–593.
4. Neutra MR, Kozlowski PA. 2006. Mucosal vaccines: the promise and the challenge. *Nat. Rev. Immunol.* 6:148–158.
5. Mowat AM. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat. Rev. Immunol.* 3:331–341.
6. Holmgren J, Czerkinsky C. 2005. Mucosal immunity and vaccines. *Nat. Med.* 11:S45–S53.
7. De Magistris MT. 2006. Mucosal delivery of vaccine antigens and its advantages in pediatrics. *Adv. Drug Deliv. Rev.* 58:52–67.
8. Kiyono H, Fukuyama S. 2004. NALT- versus Peyer's-patch-mediated mucosal immunity. *Nat. Rev. Immunol.* 4:699–710.
9. Neutra MR, Pringault E, Kraehenbuhl JP. 1996. Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Annu. Rev. Immunol.* 14:275–300.
10. Lasaro MO, Ertl HC. 2009. New insights on adenovirus as vaccine vectors. *Mol. Ther.* 17:1333–1339.
11. Liu MA. 2010. Immunologic basis of vaccine vectors. *Immunity* 33:504–515.
12. Lin SW, Cun AS, Harris-McCoy K, Ertl HC. 2007. Intramuscular rather than oral administration of replication-defective adenoviral vaccine vector induces specific CD8⁺ T cell responses in the gut. *Vaccine* 25:2187–2193.
13. Mercier GT, Nehete PN, Passeri MF, Nehete BN, Weaver EA, Templeton NS, Schluns K, Buchl SS, Sastry KJ, Barry MA. 2007. Oral immunization of rhesus macaques with adenoviral HIV vaccines using enteric-coated capsules. *Vaccine* 25:8687–8701.
14. Wang L, Cheng C, Ko SY, Kong WP, Kanekiyo M, Einfeld D, Schwartz RM, King CR, Gall JG, Nabel GJ. 2009. Delivery of human immunodeficiency virus vaccine vectors to the intestine induces enhanced mucosal cellular immunity. *J. Virol.* 83:7166–7175.
15. Thorner AR, Vogels R, Kaspers J, Weverling GJ, Holterman L, Lemckert AA, Dilraj A, McNally LM, Jeena PM, Jepsen S, Abbink P, Nanda A, Swanson PE, Bates AT, O'Brien KL, Havenga MJ, Goudsmit J, Barouch DH. 2006. Age dependence of adenovirus-specific neutralizing antibody titers in individuals from Sub-Saharan Africa. *J. Clin. Microbiol.* 44:3781–3783.
16. Barouch DH, Kik SV, Weverling GJ, Dilan R, King SL, Maxfield LF, Clark S, Ng'ang'a D, Brandariz KL, Abbink P, Sinangil F, de Bruyn G, Gray GE, Roux S, Bekker LG, Dilraj A, Kibuuka H, Robb ML, Michael NL, Anzala O, Amorinkul PN, Gilmour J, Hural J, Buchbinder SP, Seaman MS, Dolin R, Baden LR, Carville A, Mansfield KG, Pau MG, Goudsmit J. 2011. International seroepidemiology of adenovirus serotypes 5, 26, 35, and 48 in pediatric and adult populations. *Vaccine* 29:5203–5209.
17. Shinozaki T, Araki K, Ushijima H, Fujii R. 1987. Antibody response to enteric adenovirus types 40 and 41 in sera from people in various age groups. *J. Clin. Microbiol.* 25:1679–1682.
18. Kidd AH, Chroboczek J, Cusack S, Ruigrok RW. 1993. Adenovirus type 40 virions contain two distinct fibers. *Virology* 192:73–84.
19. de Jong JC, Wigand R, Kidd AH, Wadell G, Kapsenberg JG, Muzerie CJ, Wermenbol AG, Firtzlaff RG. 1983. Candidate adenoviruses 40 and 41:

- fastidious adenoviruses from human infant stool. *J. Med. Virol.* 11:215–231.
20. Tiemessen CT, Kidd AH. 1994. Adenovirus type 40 and 41 growth in vitro: host range diversity reflected by differences in patterns of DNA replication. *J. Virol.* 68:1239–1244.
 21. Yamasaki S, Miura Y, Brown E, Davydova J, Yamamoto M. 2010. Development of a method for an effective amplification of human adenovirus 40. *Arch. Virol.* 155:1059–1068.
 22. Yamasaki S, Miura Y, Davydova J, Vickers SM, Yamamoto M. 2013. Intravenous genetic mesothelin vaccine based on human adenovirus 40 inhibits growth and metastasis of pancreatic cancer. *Int. J. Cancer* 133:88–97.
 23. Worbs T, Bode U, Yan S, Hoffmann MW, Hintzen G, Bernhardt G, Förster R, Pabst O. 2006. Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. *J. Exp. Med.* 203:519–527.
 24. Brandt EB, Strait RT, Hershko D, Wang Q, Muntel EE, Scribner TA, Zimmermann N, Finkelman FD, Rothenberg ME. 2003. Mast cells are required for experimental oral allergen-induced diarrhea. *J. Clin. Invest.* 112:1666–1677.
 25. Green TP, Johnson DE, Marchessault RP, Gatto CW. 1988. Transvascular flux and tissue accrual of Evans blue: effects of endotoxin and histamine. *J. Lab. Clin. Med.* 111:173–183.
 26. Lange S, Delbro DS, Jennische E. 1994. Evans blue permeation of intestinal mucosa in the rat. *Scand. J. Gastroenterol.* 29:38–46.
 27. Zhang M, Angata T, Cho JY, Miller M, Broide DH, Varki A. 2007. Defining the in vivo function of Siglec-F, a CD33-related Siglec expressed on mouse eosinophils. *Blood* 109:4280–4287.
 28. Roy K, Mao HQ, Huang SK, Leong KW. 1999. Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. *Nat. Med.* 5:387–391.
 29. Albinsson B, Kidd AH. 1999. Adenovirus type 41 lacks an RGD alpha(v)-integrin binding motif on the penton base and undergoes delayed uptake in A549 cells. *Virus Res.* 64:125–136.
 30. Tiemessen CT, Kidd AH. 1995. The subgroup F adenoviruses. *J. Gen. Virol.* 76:481–497.
 31. Nakamura T, Sato K, Hamada H. 2003. Reduction of natural adenovirus tropism to the liver by both ablation of fiber-coxsackievirus and adenovirus receptor interaction and use of replaceable short fiber. *J. Virol.* 77:2512–2521.