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 mucosal 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.
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 µl 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 percutaneously 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 µl 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), jejenum 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 PP (visible PP 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 rAd40pΔE1-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 horsaiderish peroxidase (HRP) (Dako, Glostrup, Denmark). Tissues were fixed with 4% paraformaldehyde (PFA) in PBS, embedded in paraffin wax, sectioned at 3 µm, 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 ×100, ×200, and ×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 µm) 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) (×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 scalps, and then passed through 70- and 40-µm strainers. Splenocytes were prepared by passing through a 100-µm 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-µm strainer. Mononuclear cells were prepared in lymphocyte separation medium (Mediatech, Inc.) and passed through 40-µm 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 × 10^6) or splenocytes (>1 × 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 µg/ml of Ova peptide (SLIIFEKL, H-2Kb; ProImmune), Pan02-Ova cell lysate (generated by four freeze-thaw cycles, passed through a 0.22-µm 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 µg/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 × 10^6 events were acquired on a FACS Calibur 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 supernatant, 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 rAd40pΔE1-CMV-Ova and rAd5ΔE1-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 μL PBS/complete Freund’s adjuvant emulsion containing 300 μg Ova protein (A-5503; Sigma-Aldrich) into the tail base. Fourteen days after immunization, the mice were challenged by s.c. administration of 50 μg Ova in 20 μL PBS into the right ear pinna, while 20 μL 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.

**Diarrhoea model.** Diarrhoea and other allergic parameters were compared among control (PBS) mice and BALB/c mice pretreated with 10^10 VP of rAd40pΔE1-CMV-Ova- and rAd5ΔE1-CMV-Ova at 14 days after i.d. rAd in allergic diabetes 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 μg 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 μL PBS every 2 days, which was continued until there were two observations of diarrhoea 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. Diarrhoea was assessed by visually monitoring mice for up to 1 h following i.g. challenge. Mice demonstrating liquid stool were recorded as diarrhoea-positive animals. At the second occurrence of diarrhoea in every control mouse, plasma and feces were extracted for ELISA at 1 h after the final intrastracal (i.g.) Ova challenge.

**Systemic anaphylaxis, intestinal vascular permeability, and eosinophil counts.** Forty-eight hours after final i.g. challenge in allergic diabetes models, BALB/c mice were immunized Ova 100 μg/mouse, for systemic anaphylaxis in Evan’s blue dye (EBD) (20 mg/kg, for intestinal vascular permeability assay) in PBS (200 μL) 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 at 24 h at 30°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 (×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 rAd40pΔE1-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 rAd40-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 rAd40pΔE1-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 rAd40pΔE1-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. rAd40pΔE1-CMV-Ova (rAd40-
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

| Sample type                  | No. of mice with transgene expression/total no. of mice for: |
|------------------------------|-------------------------------------------------------------|
|                              | Control          | rAd40-Luc | rAd5-Luc |
| Jejunum without PPs          | 0/9*             | 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      |

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

**Inhibition of ovalbumin-induced delayed-type hypersensitivity response.** To evaluate whether it was possible to induce
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-\(\gamma\)-secreting CD8\(^+\) T cells to CD8\(^+\) T cells and IFN-\(\gamma\)-secreting CD4\(^+\) T (IFN-\(\gamma\)-CD3\(^e\)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 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.
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 hypersensi-
tivity, 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 co-

tons 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 exhibited markedly red co-
nels after anaphylactic shock, and bloody diarrhea was observed
in the jejunum.

Inhibition of ovalbumin-induced systemic anaphylaxis. The
effect of pretreatment on severe systemic allergic reaction was an-
alyzed 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 symp-
toms 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 sim-
ilar 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-pre-

treated mice were higher than in control mice (Fig. 5C).

| 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                                  |

TABLE 2 Anaphylactic symptom score
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 adeno-virus 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 vaccine 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.

**ACKNOWLEDGMENTS**

We thank Kazunori Aoki (National Cancer Center Research Institute, Tokyo, Japan), Hiroaki Ikeda and Hiroshi Shiku (Mie University Graduate School of Medicine, Tsu, Japan), and Ashok K. Saluja and Veena Sangwan (University of Minnesota) for their helpful discussions and Eric Brown and Stephen R. Nelson for their assistance with the manuscript.

This work was partly supported by NIH grants DK63615, CA168448, CA94084, and P50CA101955 (project 4) to M.Y.

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