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A Murine Model of Enterohemorrhagic *Escherichia coli* O157:H7 Infection to Assess Immunopotentiating Activity of Drugs on Mucosal Immunity: Effect of Drugs

Keiji Nagano[1], Takeshi Sugisaki[1], Kazuki Taguchi[1], Takumi Hara[1], Mitsuru Naiki[2] and Hiroshi Mori[1,*]

[1]*Laboratory of Microbiology, Department of Public Health Pharmacy, Gifu Pharmaceutical University, 5-6-1 Mitahora-higashi, Gifu 502-8585, Japan*

[2]*Institute of Bio-Active Science, Nippon Zoki Pharmaceutical Company, Yashiro-cho, Hyogo 673-1461, Japan*

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Abstract. An enterohemorrhagic *Escherichia coli* (EHEC) O157 oral infection murine model was established to examine the potentiating activity of drugs on mucosal immune responses. Groups of ICR mice inoculated intragastrically with 10^{11} CFU/kg EHEC O157 showed chronic intestinal infection with the pathogen that persisted over 3 weeks and resulted in the synthesis of relatively high levels of antigen specific fecal IgA antibody. Intraperitoneal administration of 80 NU/kg Neurotropin, an immunopotentiator, augmented the antigen specific mucosal immune responses to EHEC O157. On the other hand, FK506 clearly suppressed the response. To further document the augmenting effect of Neurotropin on mucosal immune responses, mice were immunized intranasally with a mixture of ovalbumin and cholera toxin. Co-administration of 80 NU/kg Neurotropin significantly potentiated the synthesis of fecal IgA and serum IgG antibodies. These results suggest that Neurotropin has potential as a mucosal adjuvant to promote secretory IgA antibody production and that the mice model of oral infection with EHEC O157 is useful for immunopharmacological studies of bacterial infection-defensive mucosal immune responses.

Keywords: Neurotropin, immunopotentiator, mucosal immunization, enterohemorrhagic *Escherichia coli* O157, murine model

Introduction

Most microbial pathogens colonize mucosal tissues such as in the gut, nasal cavity, lung, and genitalia and /or invade the host body through the mucosa to establish infections (1). Mucosal immunity functions as the primary site for host defense and serves as a critical barrier against pathogens. One major effector system for mucosal immunity is the secretory IgA (S-IgA) antibody, which is selectively transported by a receptor-mediated mechanism to external secretion and functions to eliminate pathogens by inhibiting their adherence and colonization (2). Thus, vaccines that induce pathogen specific S-IgA antibodies within the mucosa have been proposed as important for the prevention of mucosal infection (3, 4). However, conventional parenteral vaccines are generally poor stimulators of mucosal immune responses, although they can induce a degree of systemic immunity. Direct application, oral or intranasal administration, of vaccine antigens to mucosal surfaces has so far proved to be the most effective way to induce specific S-IgA antibodies. Even so, there are few effective mucosal vaccines that can induce protective immunity through mucosal immunization. Hence, various agents including cholera toxin (CT) of *Vibrio cholerae* and heat-labile enterotoxin (LT) of *Escherichia coli* have been utilized as mucosal adjuvants to induce specific S-IgA antibodies within mucosa (5). CT and LT are relatively toxic molecules, and attempts have been made to reduce their toxicity by the creating recombinant forms of these molecules. The B subunit pentamer moiety of CT has been reported in a number of clinical trials (6 – 9). In spite of these efforts, no clinically avail-

*Corresponding author. FAX: +81-058-237-5979
E-mail: morih@gifu-pu.ac.jp

able mucosal adjuvants and/or vaccines have yet been found to promote S-IgA antibody production. One reason for the lack of development of these agents is thought to be that there has been only limited use of microbial infection animal models of well-characterized S-IgA antibody production, although there have been many reports of infection models to examine the pathogenicity of microbes.

In the present study, we examined S-IgA antibody production in mice infected orally with enterohemorrhagic *E. coli* (EHEC) O157 to establish a murine model to assess the immunopotentiating activity of drugs. Humans are exposed to EHEC O157 orally, and colonization of the colon follows. Diseases induced by EHEC O157 are characterized by diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (10 – 12). The adherence of EHEC to intestinal epithelial cells is believed to be the first and an indispensable step for developing these disease manifestations. Adhesion is mediated through a type III secretion system, resulting in histological changes of the epithelial cells, which has been termed an attaching-and-effacing (A/E) lesion that includes F-actin aggregation (11, 12). We previously found that mice infected orally with EHEC O157 showed fecal shedding of EHEC O157 that persisted for over 3 weeks and that adhesion and colonization of EHEC O157 in the cecum of the mice were dependent on the type III secretion system (13). We also reported that children infected with EHEC O157 secreted S-IgA antibody specific to the bacteria into their stools (14). In the present study, we demonstrate that mice infected with EHEC O157 secrete S-IgA antibody into their feces, as do humans.

Neurotropin, non-protein fraction extracted from the inflamed skin of rabbit inoculated with the vaccinia virus, has been clinically used in Japan for chronic pain (15) and pruritus (16, 17) therapies. Previous studies have also documented the activity of Neurotropin to reconstitute immune responses of mice suppressed by immunosuppressive agents (18 – 20), stress (21, 22), and aging (23). Tomoda et al. (24) examined the effect of Neurotropin on intestinal flora of mice, and reported that Neurotropin suppressed the cyclophosphamide- and/or prednisolone-induced increase of *Candida* infection incidence within the intestine, which was argued to be secondary to enhancement of the intestinal immune responses by Neurotropin. Neurotropin is also reported to be a potentiator for T cells (19, 25).

Here, we examined Neurotropin for its mucosal adjuvant activity in two experimental models of mice, which produced specific IgA antibodies after being orally infected with EHEC O157 and/or after being immunized intranasally with ovalbumin (OVA) mixed with CT.

Finally, we detail that the murine model of EHEC O157 infection is useful for immunopharmacological studies, as exemplified by our study of the effects of Neurotropin and FK506 on mucosal immune responses.

Materials and Methods

Mice

Female ICR mice were obtained from Japan SLC, Inc. (Hamamatsu) and used after one-week of acclimatization. Mice were subjected to EHEC O157 infection experiments at 5 weeks of age and OVA immunization experiments at 7 weeks of age. Mice were maintained in the Laboratory for Animal Experiments, Gifu Pharmaceutical University, with free access to sterilized Charles River solid rodent chow (CRF-1; Oriental Yeast, Tokyo) and water (Milli-Ro water; Millipore Corporation, Bedford, MA, USA). Infectious experiments were performed in an isolation chamber for animals (Toyoriko Co., Ltd., Tokyo) and the mice were kept on stainless wire mesh to prevent them from being infected by fecal material. For the OVA immunization experiments, mice were kept in cages in a filtered lamina airflow chamber. These animal experiments were performed in accordance with the standards listed in the Guidance for the Care and Use of Laboratory Animals of Gifu Pharmaceutical University.

Bacterial strains

EHEC O157 (strain GPU96MM, serotype O157:H7, producing Stx1 and Stx2) was a clinical isolate from stool samples of a patient with hemorrhagic colitis collected during an outbreak of disease in Gifu, Japan, in 1996. *E. coli* K12 (strain C600) was used to deplete naturally occurring antibodies in samples prior to their assay for antibody titers against EHEC O157 whole cells.

Intragastrical inoculation of EHEC O157

EHEC O157 stored at −80°C were seeded into nutrient broth (Nissui Pharmaceutical Co., Ltd., Tokyo) and cultured statically for 15 h at 37°C. The cultures were diluted 1:100 in the same medium and incubated for a further 4 h at 37°C. The bacteria were harvested during logarithmic growth by centrifugation for 20 min at 2,000 × *g* and then resuspended in sterilized phosphate-buffered saline (PBS, pH 7.4). The bacterial concentration (colony forming unit (CFU)/ml) was determined by measurement of the optical density (OD) at 600 nm according to a standard curve prepared previously and then diluted to an appropriate concentration with PBS. Mice were starved of food for 8 h, and then they were injected i.p. with 25 mg/kg cimetidine. The bacterial

suspension was inoculated into the mice intragastrically at a volume of 0.1 ml/10 g body weight 15 min after the cimetidine injection.

Intranasal immunization with OVA

Lightly anesthetized mice were given a mixture containing 25 mg/ml OVA (product No. A5503; Sigma-Aldrich Co., St. Louis, MO, USA) and 50 μg/ml CT (List Biological Laboratories, Inc., Campbell, CA, USA) via both nasal cavities at a volume of 10 μl each (total 20 μl) 4 times at weekly intervals.

Administration of Neurotropin and FK506

Neurotropin™ (Nippon Zoki Pharmaceutical Co., Ltd., Osaka) was dissolved, and FK506 (Fujisawa Pharmaceutical Co., Ltd., Osaka) was suspended in sterilized saline at the desired concentrations. The concentration of Neurotropin was expressed as the Neurotropin unit (NU), which was determined by bio-assay (26). One NU almost corresponded to 1 mg. The dilutions were administered i.p. to mice at a volume of 0.1 ml/10 g body weight. The control group was administered saline by the same route and at the same volume as utilized for drug administration.

Enumeration of EHEC O157 shed in feces

Fresh feces of mice were weighed and suspended in PBS. The fecal suspensions were then plated on EHEC O157 selective media, Sorbitor MacConkey agar (Nissui Pharmaceutical Co., Ltd.) supplemented with 20 μg/ml novobiocin and 0.1 μg/ml cefixime (Fujisawa Pharmaceutical Co., Ltd.). White colonies that developed after incubation for 24 h at 37°C were counted. The limit of detection was 10^2 CFU/g feces. A value of 10^2 CFU/g feces was assigned to any culture showing no detectable colonies for the purposes of deriving statistical data.

Extraction of antibodies from feces

To determine the kinetics of EHEC O157 specific antibody synthesis, an aliquot of fresh fecal material was suspended by vigorous agitation in a 10-fold volume of PBS containing 0.1% NaN_3 (PBS-NaN_3). The fecal suspension was centrifuged at 20,000 × *g* for 5 min, the supernatant fluid recovered and again centrifuged at 20,000 × *g* for 5 min, and the supernatant fluid once again recovered and then centrifuged at 20,000 × *g* for 15 min. The final supernatant fluid was designated as PBS-Ex-10 and assayed for the presence of antibodies by enzyme-linked immunosorbent assay (ELISA). The fecal pellets obtained following the first centrifugation step were resuspended in an acidic buffer, 0.5 M glycine-HCl buffer (pH 1.8) containing 0.1% NaN_3, in 10-fold volume of the initial fecal weight to recover antibodies that may have been complexed to EHEC O157. The supernatant fluid from this initial extraction was then subjected to the same centrifugation steps as outlined above. The acidic extract was neutralized by mixing with an equal volume of 1.0 M Tris-HCl buffer (pH 8.0) containing 0.1% NaN_3. The final fluid was termed PBS-Acid-Ex-20.

For analysis of fecal antibodies from mice infected with EHEC O157 in which the effect of Neurotropin on the fecal antibody production was assessed, antibodies were extracted with the acidic buffer, but without the PBS-NaN_3. The other steps were identical to the extraction procedure outlined above. These extracts were designated as Acid-Ex-20.

In OVA immunization experiments, fresh feces were subjected to extraction with PBS-NaN_3 at 10-fold volume of the fecal weight. The extraction and centrifugation processes were the same as described above. The resulting supernatant was designated as OVA-PBS-Ex-10.

Secondary antibodies used in ELISA

Peroxidase-conjugated goat anti-mouse IgM (mu-chain specific), goat anti-mouse IgG (gamma-chain specific), and goat anti-mouse IgA (alpha-chain specific) were purchased from Southern Biotechnology Associates, Inc., Birmingham, AL, USA.

Filtration ELISA to detect antibodies against EHEC O157 whole cells in fecal samples and sera

The procedures for pre-absorption of test samples with *E. coli* K12 and Filtration ELISA was essentially the same as described previously (14). Briefly, the washing buffer was PBS containing 0.1% Tween 20. Blocking buffer-A consisted of PBS containing 1% bovine serum albumin (BSA, Fraction V; Nacalai Tesque, Inc., Kyoto), 5% normal calf serum (NCS; Irvin Scientific, Kogan, UT, USA), 0.1% Tween 20, and 0.1% NaN_3. Blocking buffer-B consisted of PBS containing 1% BSA, 5% NCS, 0.1% Tween 20, and 0.01% thimerosal.

EHEC O157 and/or *E. coli* K12 cultured in nutrient broth were washed with PBS-NaN_3 by centrifugation, and the number of bacterial cells suspended in PBS-NaN_3 was determined using a Petroff-Hausser hemocytometer for bacteria (Sunlead Glass Co., Tokyo) with gentian violet stain.

Fecal extracts and serum samples were pre-absorbed with *E. coli* K12 to remove natural antibodies just before subjecting them to Filtration ELISA. *E. coli* K12 (2×10^7 cells/well) were applied over the filter of the filtration plate wells (MultiScreen®-GV, 96-well membrane plate, pore size: 0.22 μm; Millipore Corporation).

The suspending buffer was removed by vacuum filtration through a filter with a commercially available filtration unit (MAVM09601, Millipore Corporation). Then, the remaining cells were suspended in wells with 150 μl of fecal extracts (PBS-Ex-10, PBS-Acid-Ex-20, and Acid-Ex-20 at 5-, 2.5-, and 5-fold dilutions with blocking buffer-A, respectively) or sera (a 20-fold dilution with blocking buffer-A), followed by incubation for 2 h at 37°C. The solutions in the wells were passed through the membrane by vacuum and transferred to another plate settled under the filtration plate. The absorbed solutions were diluted 2-fold with blocking buffer-A to be assayed by Filtration ELISA.

Filtration ELISA was performed as follows: A suspension of 10^8 cells/ml EHEC O157 in a volume of 200 μl was applied over the filter of the filtration plate well. After removing the suspending buffer, the cells were incubated with 200 μl of blocking buffer-A supplemented with 0.4 μl/ml of 30% H_2O_2 at 37°C for 1 h to inactivate endogenous cellular peroxidases. For this assay, the bacterial cells utilized as an antigen were washed by vacuum filtration through a filter and resuspended in washing buffer with a microplate shaker. Fecal extracts and sera, 100 μl of each, were added to the bacterial cells at a 10-fold final dilution of PBS-Ex-10, 5-fold of PBS-Acid-Ex-20, 10-fold of Acid-Ex-20, and 40-fold of sera; and then the cells were incubated for 2 h at 37°C. After the incubation, the cells were washed 4 times and incubated with peroxidase-labeled secondary antibodies diluted 1,000-fold with blocking buffer-B. After washing 5 times, the wells were subjected to the enzymatic reaction for 30 min at 37°C with 150 μl substrate solution (pH 5.0) consisting of 0.05 M citric acid, 0.1 M disodium hydrogen phosphate, 0.4 mg/ml of *o*-phenylenediamine, and 0.4 μl/ml of 30% H_2O_2. The reaction was terminated by adding 50 μl of 2 M H_2SO_4. Aliquots (100 μl) of the resulting solution were transferred to transparent 96-well flat-bottom micro-titer plates. The OD at 492 nm (reference 660 nm) was measured in the transferred solution with a microplate-reader (MTP-120; Corona Electric Co., Ibaragi). To examine background OD values, blocking buffer-A was used in place of test samples. All assays were carried out in duplicate, and the average of duplicate OD measurements at the defined dilution of test samples was considered the antibody titer.

ELISA to detect antibodies against O157-LPS

Lipopolysaccharide of EHEC O157 (O157-LPS) was extracted from *E. coli* GPU96MM by the hot-phenol method (27, 28). The phenol layer so obtained was used for purification, because our preliminary experiments showed that O157-LPS moved to the phenol layer but not the aqueous layer, in agreement with Westerman et al. (28). O157-LPS was purified by repeating centrifugation at 80,000 × *g* at 4°C for 4 h to remove proteins. The concentration of O157-LPS was determined by assaying for 2-keto-3-deoxyoctonic acid (KDO) (29). The assay of antibodies against O157-LPS was performed by standard ELISA (30). Briefly, Immulon 1B plates (Dynex Technologies, Inc., Chantilly, VA, USA) were coated with 0.12 μg KDO/well of O157-LPS diluted with PBS. The buffers and substrate solution used in the assay were essentially the same as those used in Filtration ELISA. Fecal extracts of Acid-Ex-20 and sera were applied to the wells at 5- and 20-fold, respectively, dilutions with blocking buffer-A and incubated for 2 h at 37°C. The secondary antibodies were applied to the wells at a 1,000-fold dilution with blocking buffer-B. The enzymatic reaction at 37°C was stopped after 30 min with 2 M H_2SO_4 and OD at 492 nm (reference 660 nm) was measured with a microplate-reader. To examine background OD values, blocking buffer-A was used in place of test samples. All assays were carried out in duplicate, and the average of duplicate OD measurements at the defined dilution of test samples was considered the antibody titer.

ELISA to detect antibodies against OVA

The assay was performed by standard ELISA (30). Briefly, Nunc-Immuno plates (MaxiSorp Surface; Nalge-Nunc International, Rochester, NY, USA) were coated with 1.0 μg/well of OVA dissolved in carbonate buffer (pH 9.6). Blocking and sample dilution buffers were Casein buffer-A consisting of 1% casein, 0.1% NaN_3, and PBS. Washing buffer was the same as used in Filtration ELISA. Fecal samples of OVA-PBS-Ex-10 were applied to the wells at a 5-fold dilution for detecting IgA antibodies. Test sera were applied to the wells at 50-, 2,000-, and 200-fold dilutions for detecting IgM, IgG, and IgA antibodies, respectively. Secondary antibodies were applied to the wells at a 1:1,000 dilution with Casein buffer-B consisting of 1% casein, 0.01% thimerosal, and PBS. Other procedures were the same as in the ELISA to detect antibodies against O157-LPS.

Statistical analyses

The results were expressed as means ± S.E.M. If the null hypothesis of the homogeneity of variance by the Bartlett test was accepted, the parametric Duncan's multiple range test after one-way analysis of variance (ANOVA) was used to assess the statistical significance of differences between the control and drug-treated groups. If the null hypothesis of the homogeneity of variance by the Bartlett test was not accepted, the data were ranked and subjected to the Kruskal-Wallis test

and then subjected to the non-parametric Duncan's multiple range test (ranked multiple range test) to analyze the significance of differences between the control and the drug-treated groups. A value of $P<0.05$ was considered a significant difference in all statistical analyses.

Results

Kinetics of fecal shedding of EHEC O157 and antibody production against EHEC O157 whole cells

Fecal shedding of EHEC O157 and fecal IgM, IgG, and IgA antibodies against EHEC O157 whole cells were examined at 1, 2, 3, and 4 weeks after intragastric inoculation with 10^9, 10^{10}, and 10^{11} CFU/kg EHEC O157 in mice.

The number of EHEC O157 shed in the feces of the 10^9 CFU/kg group was less than those of the 10^{10} and 10^{11} CFU/kg groups at 1 and 2 weeks after inoculation (Fig. 1). The 10^{10} and 10^{11} CFU/kg groups shed comparable numbers of bacteria at 1 and 2 weeks. While the number of bacteria in the mice receiving 10^{10} CFU/kg decreased greatly at 4 weeks, the number in the 10^{11} CFU/kg group had decreased by 3 weeks.

Antibody titers against EHEC O157 whole cells in feces were examined at the same final dilution for PBS extracts (PBS-Ex-10) and acidic buffer extracts (PBS-Acid-Ex-20). IgM and IgG antibodies were not detected in either PBS-Ex-10 or PBS-Acid-Ex-20 throughout the experimental period (data not shown). However, IgA antibody was detected in both PBS-Ex-10 and PBS-Acid-Ex-20 of all groups from 2 or 3 weeks after inoculation (Fig. 2). The antibody titer in PBS-Ex-10 of the 10^{11} CFU/kg group peaked at 3 and 4 weeks and was higher than those of the other two groups. Time courses of IgA antibody titers in PBS-Acid-Ex-20 were comparable to those of PBS-Ex-10 in the 10^9 CFU/kg and 10^{10} CFU/kg groups. On the other hand, the IgA antibody titer in PBS-Acid-Ex-20 of the 10^{11} CFU/kg group decreased at 4 weeks, contrary to the same level of titer in PBS-Ex-10 being kept from 3 to 4 weeks.

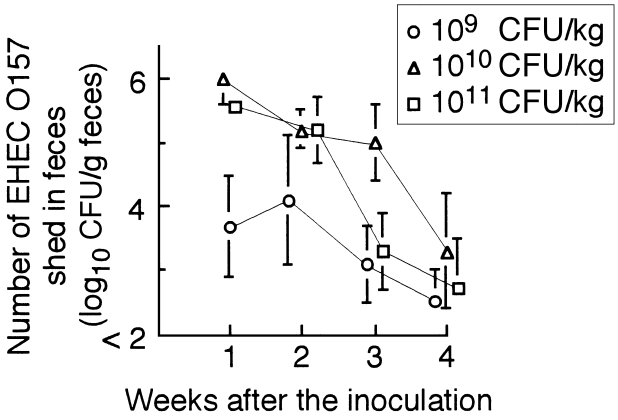

Fig. 1. Fecal shedding of EHEC O157 in mice inoculated intragastrically with 10^9, 10^{10}, and 10^{11} CFU/kg of the bacteria. Each point shows the mean ± S.E.M. of 4 to 6 mice.

Effect of Neurotropin and FK506 on fecal shedding of the bacteria, and serum and fecal antibody production in mice infected with EHEC O157

Mice were inoculated intragastrically with 10^{11} CFU /kg of EHEC O157. Fecal samples were collected at 3, 4, and 5 weeks after inoculation to examine the fecal shedding of EHEC O157 and IgA antibody titers against

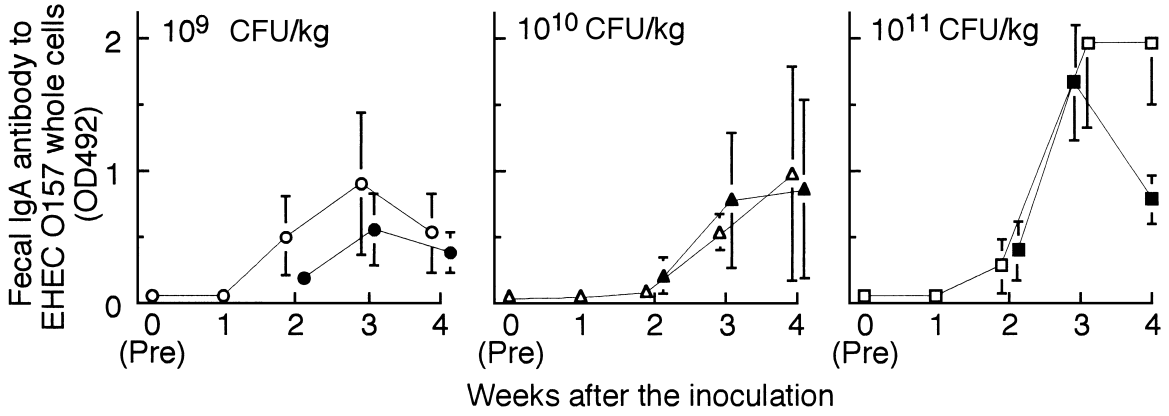

Fig. 2. Fecal IgA antibody titer against EHEC O157 whole cells in mice inoculated intragastrically with 10^9 (left panel), 10^{10} (middle panel), and 10^{11} (right panel) CFU/kg of the bacteria. Open and closed symbols show the antibody titer in fecal samples extracted with PBS (PBS-Ex-10) and the acidic buffer (PBS-Aid-Ex-20) that followed, respectively. The antibody titers were assayed at a 10-fold final dilution of PBS-Ex-10 and at a 5-fold final dilution of PBS-Acid-Ex-20. Each point shows the mean ± S.E.M. of 4 to 6 mice.

EHEC O157 whole cells and O157-LPS. Sera were collected from mice at 5 weeks after inoculation to examine IgM, IgG, and IgA antibody titers against EHEC O157 whole cells and O157-LPS. Neurotropin at doses of 50, 80, and 100 NU/kg and FK506 at a dose of 3 mg/kg were administered i.p. daily from 5 days before the bacterial inoculation to the day of the final sample collection.

There was no significant difference in the fecal shedding of EHEC O157 between the control group and the Neurotropin groups at any of the doses at 3, 4, and 5 weeks after the inoculation (Fig. 3, top panel). Fecal shedding in the FK506 group was not significantly different from those in the control group at any weeks after inoculation, although the shedding in the FK506 group showed a tendency to be less than that of the control group at 5 weeks.

The fecal IgA antibody titer against EHEC O157 whole cells (Fig. 3, middle panel) peaked at 4 weeks after inoculation in the control group. In comparison with the control group, the titer at 3 weeks was significantly higher in the group of mice administered 80 NU /kg Neurotropin. On the other hand, the titers at 3, 4, and 5 weeks were significantly lower in mice administered

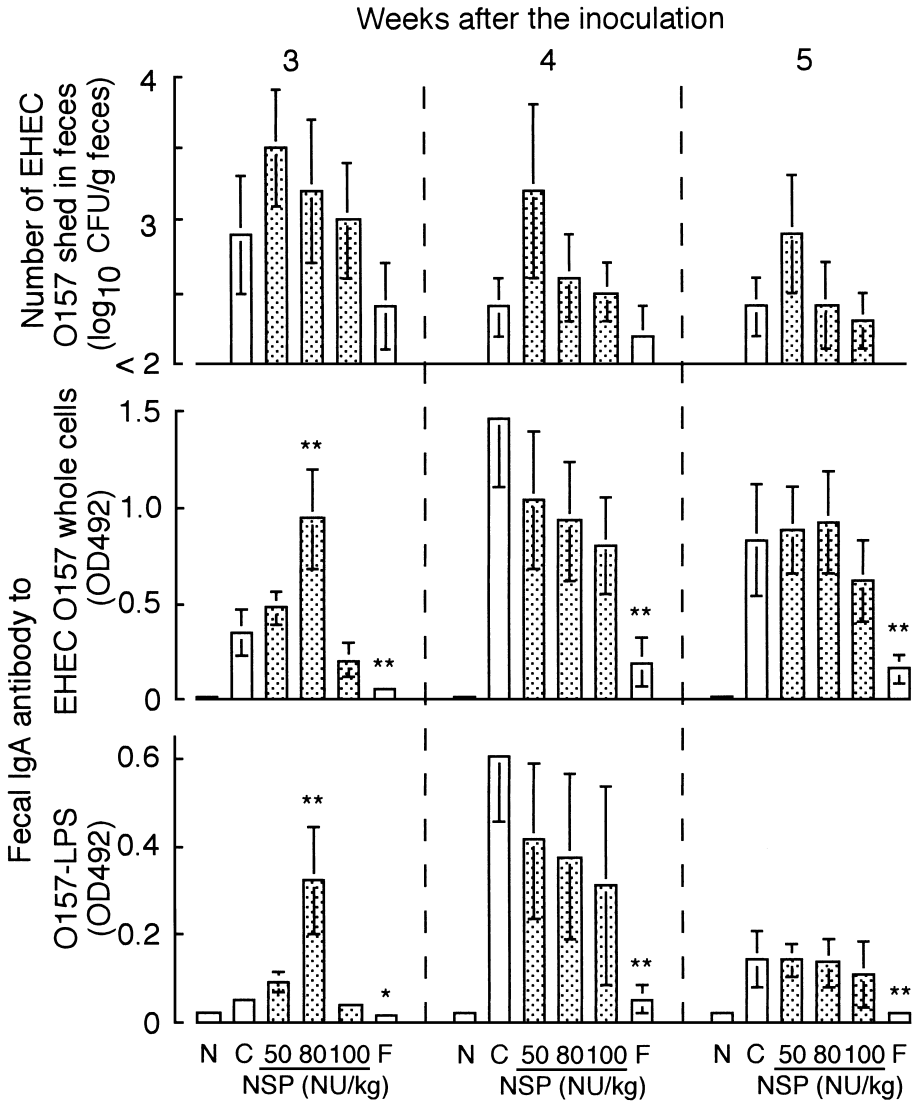

Fig. 3. Effect of Neurotropin on fecal shedding of EHEC O157 (top panel), and fecal antibody titers against EHEC O157 whole cells (middle panel) and O157-LPS (bottom panel) in mice inoculated intragastrically with 10^{11} CFU/kg EHEC O157. Mice received Neurotropin i.p. at doses of 50, 80, and 100 NU/kg per day or FK506 at a dose of 3 mg/kg per day consecutively from 5 days before bacterial inoculation. Antibody titers against EHEC O157 whole cells and O157-LPS were examined at a 10-fold dilution and a 5-fold dilution of Acid-Ex-20, respectively. N: normal, C: control, F: FK506. Each column shows the mean ± S.E.M. of 7 mice. *, **: Statistically significant difference from the controls at $P<0.05$ and $P<0.01$, respectively.

FK506.

The fecal IgA antibody titer against O157-LPS in the control group (Fig. 3, bottom panel) also peaked at 4 weeks after. The antibody titer in mice administered 80 NU/kg Neurotropin was significantly higher than that of the control group at 3 weeks.

Serum IgM, IgG, and IgA antibody titers against EHEC O157 whole cells and O157-LPS at 5 weeks after inoculation were clearly higher in the control group than those in the non-infected (normal) group (Fig. 4). IgM antibody titers against EHEC O157 whole cells and O157-LPS in each of the dosage levels of the Neurotropin groups and the FK506 group were all higher than those of the control group, with or without statistical significance. The IgG and IgA antibody titers in each of the dose of the Neurotropin groups were not significantly different than the control group. In contrast, IgG and IgA antibody titers in the FK506 group were significantly lower compared with the control group.

Effect of Neurotropin on fecal and serum antibody production in mice immunized with OVA

Mice were immunized intranasally with OVA 4 times at weekly intervals in combination with CT. Feces and sera were collected 1 week after the last immunization and examined for OVA-specific IgM, IgG, and IgA antibodies. Neurotropin was administered daily at a dose of 80 NU/kg to mice from 5 days before the first OVA immunization until the day of fecal and serum sample collections.

The fecal IgA antibody titer of the Neurotropin group was significantly higher than that of the control group, while the serum IgA antibody titer of the Neurotropin group showed a tendency to be higher than that of the control group (Fig. 5). The serum IgM antibody titer of the Neurotropin group showed no difference from that of the control group, but the serum IgG antibody titer of the Neurotropin group was significantly higher than that of the control group.

Discussion

When mice were inoculated intragastrically with 10^{10} and 10^{11} CFU/kg EHEC O157, fecal shedding of the bacteria was detected at 10^5 to 10^6 CFU/g feces over 2 weeks and decreased from more than 3 weeks after

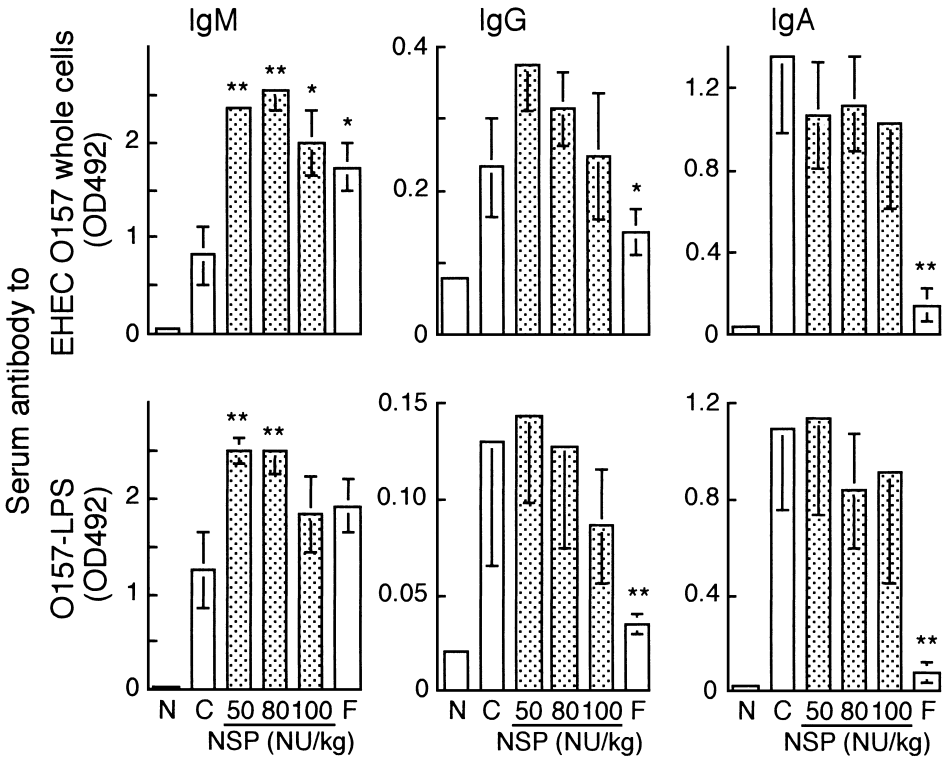

Fig. 4. Effect of Neurotropin on serum antibody titers against EHEC O157 whole cells (upper panel) and O157-LPS (lower panel) in mice at 5 weeks after intragastrical inoculation of 10^{11} CFU/kg EHEC O157. See legend of Fig. 3 for the protocol for Neurotropin and FK506 treatments. Antibody titers were examined at a 40-fold dilution. N: normal, C: control, F: FK506. Each column shows the mean ± S.E.M. of 7 mice. *, **: Statistically significant difference from the controls at $P<0.05$ and $P<0.01$, respectively.

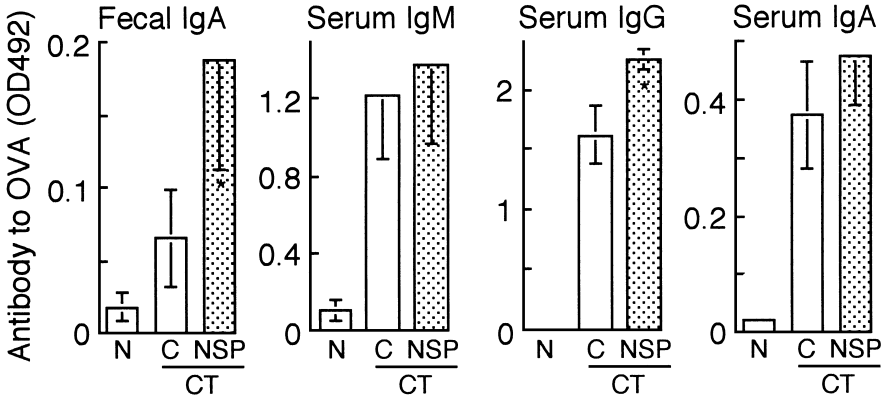

Fig. 5. Effect of Neurotropin on fecal IgA, and serum IgM, IgG and IgA antibody titers to ovalbumin (OVA). Mice were immunized intranasally with a mixture of 500 μg OVA and 1.0 μg of CT 4 times at weekly intervals and received 80 NU/kg per day of Neurotropin i.p. consecutively from 5 days before the first immunization with OVA. Feces and serum were collected at one week after the last immunization. The fecal IgA antibody titers were examined at a 5-fold dilution of OVA-PBS-Ex-10, and serum IgM, IgG, and IgA antibody titers at 50-, 2,000-, and 200-fold dilutions of the sera, respectively. N: normal, C: control. Each column shows the mean ± S.E.M. of 6 mice. *: Statistically significant difference from the controls at $P<0.05$.

inoculation (Fig. 1). Fecal IgA antibody titer began to increase following 2 weeks after inoculation in all groups, and it showed peak high titers at 3 weeks (Fig. 2). The antibody titers of the 10^{11} CFU/kg group were relatively higher in comparison with any other group, and this dose might be optimal for the production of specific S-IgA in the intestine. Antibody titers re-extracted with the acidic buffer from feces after extracting with PBS were almost comparable to those extracted with PBS except for the lower titer of acidic re-extraction at 4 weeks of mice infected with 10^{11} CFU/kg EHEC O157 (Fig. 2). It is probable that the lower titer of the acidic extract at 4 weeks was dependent on the decrease of the bacterial shedding and the resulting decrease of antibodies complexed to the bacteria. These results indicated that it is desirable to extract free and EHEC O157-bound antibodies from fecal samples with an acidic buffer to evaluate the total amount of antibody secreted. In the following experiment of oral infection, antibodies were extracted with the acidic buffer without PBS extraction.

Mice were infected with 10^{11} CFU/kg EHEC O157 and the IgA antibody titers were evaluated at 3, 4, and 5 weeks after infection by directly extracting the antibody from their feces with acidic buffer to examine the effect of Neurotropin and/or FK506 on the mucosal immune response.

In the control group, fecal IgA antibody titer peaked at 4 weeks and decreased greatly at 5 weeks, especially in IgA antibody to O157-LPS (Fig. 3). Neurotropin of 80 NU/kg increased fecal IgA antibody titers against EHEC O157 whole cells and O157-LPS significantly at 3 weeks, but not at 4 and 5 weeks. Thus, Neurotropin of 80 NU/kg enhanced the immune response and probably advanced the induction time of the IgA antibody production, although the peak time of antibody production in mice administered Neurotropin remains to be elucidated. Since mice that received 50 or 100 NU/kg Neurotropin did not show any augmented response, 80 NU/kg Neurotropin might be an optimal dose. Actually, the dose-response curve of Neurotropin has showed a bell-shaped in some experiments (19, 22, 31).

Neurotropin at any dose increased IgM antibody titer to EHEC O157 whole cells and O157-LPS, but not IgG and IgA antibody titers in serum collected at 5 weeks after infection. Many papers have reported a less invasive activity of EHEC O157 into epithelial cells (32–35), although there are conflicting results (36, 37). Not only mucosal S-IgA antibody but also systemic IgM and IgG antibodies were produced in the serum of mice, even from oral infection. There is a possibility that antigenic stimulation and the antibody production mechanisms that follow are different between mucosal S-IgA antibody production and systemic antibody production of the IgM and IgG isotypes. However, these points remain to be clarified. In the present study, we only examined the effect of Neurotropin on the serum antibody titer at 5 weeks after the infection. To clarify the effect of Neurotropin on systemic antibody production, additional experiments are required following an appropriate protocol to examine the time course of serum antibody production in mice infected orally with EHEC O157, because Neurotropin may advance the induction time of the systemic antibody production as was found in that of fecal IgA antibody.

FK506 clearly suppressed IgG and IgA serum anti-

body production as well as fecal IgA antibody production. It is known that the immunosuppressive drug FK506 impairs T cell-mediated immune responses by inhibiting the synthesis of cytokines such as interleukin-2 production (38, 39). The results suggest that the production of fecal IgA antibody in mice orally infected with the bacteria, especially against glycolipid LPS antigen, was regulated by T cells as well as the systemic IgG and IgA antibody production. The production of serum IgM antibody was increased by the administration of FK506, suggesting that the production was little regulated by T cells.

Fecal shedding of EHEC O157 decreased obviously 3 and 4 weeks after inoculation, while the fecal specific IgA antibody titer increased (Figs. 1 – 3). The specific IgA antibodies in the intestine may play some role in eliminating the pathogen from the intestine. However, Neurotropin of 80 NU/kg did not promote decrease of fecal shedding of EHEC O157, while this dose of Neurotropin advanced the induction time of fecal specific IgA antibody. Further study will be required to clarify how the specific IgA antibodies contribute to the host defense.

It is well documented that mice immunized intranasally with OVA mixed with CT produce OVA-specific IgA antibody in the intestine and serve as a model for the assessment of mucosal immune responses (40 – 42). Administration of 80 NU/kg Neurotropin to mice immunized intranasally with the mixture of OVA and CT significantly potentiated the production of fecal IgA and serum IgG antibodies. These results suggest that Neurotropin potentiates not only systemic immune but also mucosal immune responses, in accord with the results from EHEC O157 infection mice.

In the present study, we established an EHEC O157 oral infection mouse model and showed that the specific S-IgA antibody was induced in the intestine of each mouse. We further showed that 80 NU/kg Neurotropin potentiates fecal IgA antibody production in mice infected with EHEC O157 and potentiates fecal IgA and serum IgG antibody production in mice immunized intranasally with OVA and CT. These results suggest that Neurotropin has potential as a clinical available mucosal adjuvant to promote S-IgA antibody production and that the mouse infection model presented here is useful for immunopharmacological studies of the effect of drugs on the bacterial infection-defensive mucosal immune response.

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