Adhesion of Enterohemorrhagic Escherichia coli O157:H7 to the Intestinal Epithelia Is Essential for Inducing Secretory IgA Antibody Production in the Intestine of Mice

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We examined whether adherence of enterohemorrhagic Escherichia coli (EHEC) O157:H7 to intestinal epithelial cells contributed to the induction of secretory immunoglobulin A (IgA) antibody production in mice. Wild-type EHEC O157:H7 and its mutants deficient in the espA, sepL, tir and eae genes, encoding adherent factors on the locus of enterocyte effacement (LEE), were inoculated intragastrically into mice. Inoculation of wild-type EHEC induced fecal IgA antibodies specific to EHEC at 4 weeks after the inoculation, but that of espA- and sepL-deletion mutants did not. Furthermore, even 4 inoculations at weekly intervals with espA-deletion mutant, heat-killed wild-type EHEC and nonpathogenic E. coli did not induce fecal IgA antibodies, although these bacterial inoculations induced serum antibodies. Kanamycin (Km)-treated mice showed prolonged and similar fecal shedding of Km-resistant mutants of EHEC O157:H7 including A2-F6 having intact LEE, A6-E7 (sepL-insertion mutant), G1-E11 (tir-insertion mutant) and Δeae (eae-deletion mutant). In this case, A2-F6 induced fecal IgA antibodies, but the other mutants with defective LEE did not. In contrast to the fecal IgA antibodies, serum IgM and IgG antibodies were induced in mice inoculated with any of the LEE defective mutants as well as A2-F6. Thus, adhesion of EHEC to epithelial cells is essential for inducing the mucosal immune response in the intestine but not for inducing the systemic immune response.

Key words  Escherichia coli O157; bacterial adhesion; immunoglobulin A; mouse

Enterohemorrhagic Escherichia coli (EHEC) colonizes in the intestine and causes diseases such as diarrhea, hemorrhagic colitis and hemolytic uremic syndrome in humans.1,2 Adhesion of EHEC to intestinal epithelial cells may be critical for developing the diseases as well as production of Shiga toxin (Stx)-1 and/or -2. The adhesion is mainly mediated through a type III secretion system (TTSS),3 resulting in histological changes of the epithelial cells, including F-actin aggregation, which is called an attaching-and-effacing lesion.4 All the genes necessary to cause the lesion are located on the locus of enterocyte effacement (LEE).5,6 Intimin, encoded by the eae gene, is expressed on the outer membrane of EHEC as a ligand for Tir (translocated intimin receptor). Tir, encoded by the tir gene located on the LEE as well, is secreted and translocated into the host cells through the TTSS.5,6 EspA, encoded by the espA gene, constitutes TTSS and forms a hollow extracellular filament through which various proteins including Esp (E. coli secreted protein) proteins and Tir are secreted and translocated into the host cells.5,6 In addition, a sepL-deficient mutant did not secrete EspA and Tir into the culture supernatant due to defects in TTSS and lost adherent activity.5,9,10 We reported that EHEC O157:H7 adhered to and caused F-actin accumulation in the cecal epithelial cells in the specific pathogen free mice, and colonized over 3 weeks following oral inoculation of the pathogen, while espA- and sepL-deletion mutants quickly disappeared from the intestine without colonization.11 Thus, adhesion and colonization of EHEC depend on the TTSS, even in mice.

Meanwhile, there are many reports studying the immune responses against EHEC in humans12–14 and animals15,16 as host defense mechanisms. We also reported that fecal immunoglobulin A (IgA) antibodies against EHEC O157:H7 increased not only in children infected with the pathogen7 but also in mice inoculated orally.18 Camara et al.19 demonstrated that colostrum IgA antibodies against intimin of enteropathogenic E. coli, which have a LEE cluster similar to that of EHEC, inhibited the adherence of the bacteria to HeLa cells. Thus, secretory IgA (S-IgA) antibodies are transported to the mucosal surface through the epithelial cells by a receptor-mediated mechanism, and may function to eliminate the pathogen from the intestine by inhibiting the adhesion and colonization as one major effector molecule for the mucosal immunity.20 However, the induction mechanisms of the mucosal immune responses, including S-IgA antibody production, are insufficiently understood while those of the systemic immune responses, including serum antibody production, have been extensively elucidated.21

In the present study using EHEC O157:H7 mutated in the espA, sepL, tir and eae genes, we show that adherence of EHEC O157:H7 to the intestinal epithelial cells is essential for inducing S-IgA antibody production in mice.

MATERIALS AND METHODS

Mice  Female ICR mice were obtained from Japan SLC Inc. (Hamamatsu, Japan) and used at 5 weeks of age after one week of acclimatization. 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, Japan) and water (Milli-Ro water, Millipore Corporation, Bedford, MA, U.S.A.) supplemented with or without 0.5 mg/mL kanamycin sulfate (Km, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Infection experiments were carried out in an isolation chamber for animals (Toyoriko Co., Ltd., Tokyo, Japan) and the mice were kept on stainless wire mesh to prevent them from being infected by fecal material. Number of mice used in each experiment was 5 to 7 per group as indicated in individual figure legends. 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** The bacterial strains used in this study are listed in Table 1. These bacteria were stored at −80°C until use in experiments. Sakai derivatives, A2-F6, A6-E7, G1-E11 and Δeae, were grown in a medium containing 3 mg/mL Km to increase the Km-resistance and stored at −80°C. It was confirmed in advance that A2-F6 adhered to HeLa cells, formed a micro-colony and accumulated cytoskeletal F-actin in the cells beneath the adhered bacteria comparably to wild-type GPU96MM and Sakai (data not shown). The stored GPU96MM, GPU991, GPU992 and IID5208 were seeded into nutrient broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), and the A2-F6, A6-E7, G1-E11 and Δeae were seeded into nutrient broth containing 1 mg/mL Km, and cultured statically at 37°C for 15 h. The cultures were diluted 1:100 in the same medium and incubated at 37°C for a further 4 h. And then, the bacteria in logarithmic growth were used for intragastrical inoculation into mice and filtration enzyme-linked immunosorbent assay (ELISA) as antigens as described below.

**Intragastrical Inoculation of E. coli Strains** The bacteria in logarithmic growth were harvested by centrifugation at 2000×g for 20 min and then re-suspended in sterilized phosphate-buffered saline (PBS, pH 7.4). The bacterial concentration (colony-forming units (CFU)/mL) was determined by an optical density (OD) at 600 nm according to a standard curve with C600 and filtration ELISA were essentially the same as described previously. Washing buffer was PBS containing 0.1% NaN₃. The fecal suspension was centrifuged at 20000×g for 5 min. The supernatant was recovered and centrifuged again at 20000×g for 5 min, and then the supernatant was once again recovered and centrifuged at 20000×g for 15 min. The acidic extract was neutralized by mixing with an equal volume of 1.0 M Tris–HCl buffer (pH 8.0) containing 0.1% NaN₃.

**Extraction of Antibodies from Feces** Antibodies in feces were extracted with an acidic buffer according to our previous paper. Fresh feces of mice were collected and suspended in a 10-fold volume of PBS (10 µL/mg feces). To detect *E. coli* GPU96MM, 10-fold serial PBS dilutions of the suspension were plated in a volume of 100 µL on sorbitol MacConkey agar (Nissui Pharmaceutical Co., Ltd.) supplemented with 20 µg/mL of novobiocin and 0.1 µg/mL of cefixime. To detect Km-resistant mutants of GPU991, GPU992, A2-F6, A6-E7, G1-E11 and Δeae, the fecal suspensions were plated on sorbitol MacConkey agar supplemented with 50 µg/mL of Km. After incubation at 37°C for 24 h, translucent white colonies were counted. Colonies were selected randomly, and it was confirmed that the bacteria caused agglutination by *E. coli* O157 antisera (Denka Seiken Co., Ltd., Tokyo, Japan). The detection limit was 10² CFU/g feces. For the purpose of deriving statistical data, a value of 10² CFU/g feces was assigned to the culture that had shown no colonies.

**Detection of Fecal Bacteria** Fresh feces of mice were collected and suspended in a 10-fold volume of PBS (10 µL/mg feces). To detect *E. coli* GPU96MM, 10-fold serial PBS dilutions of the suspension were plated in a volume of 100 µL on sorbitol MacConkey agar (Nissui Pharmaceutical Co., Ltd.) supplemented with 20 µg/mL of novobiocin and 0.1 µg/mL of cefixime. To detect Km-resistant mutants of GPU991, GPU992, A2-F6, A6-E7, G1-E11 and Δeae, the fecal suspensions were plated on sorbitol MacConkey agar supplemented with 50 µg/mL of Km. After incubation at 37°C for 24 h, translucent white colonies were counted. Colonies were selected randomly, and it was confirmed that the bacteria caused agglutination by *E. coli* O157 antisera (Denka Seiken Co., Ltd., Tokyo, Japan). The detection limit was 10² CFU/g feces. For the purpose of deriving statistical data, a value of 10² CFU/g feces was assigned to the culture that had shown no colonies.

**Extractions and Detection of Antibodies in Feces** Antibodies in feces were extracted with an acidic buffer according to our previous paper. Fresh feces of mice were suspended by vigorous agitation in a 10-fold volume of 0.5 M glycine–HCl buffer (pH 1.8) containing 0.1% NaN₃. The fecal suspension was centrifuged at 20000×g for 5 min. The supernatant was recovered and centrifuged again at 20000×g for 5 min, and then the supernatant was once again recovered and centrifuged at 20000×g for 15 min. The acid extract was neutralized by mixing with an equal volume of 1.0 M Tris–HCl buffer (pH 8.0) containing 0.1% NaN₃.

**Filtration ELISA to Detect Antibodies against E. coli Whole Cells** The procedures for the pre-absorption of samples with C600 and filtration ELISA were essentially the same as described previously. Washing buffer was PBS containing 0.1% Tween 20. Blocking buffer-A consisted of PBS containing 1% bovine serum albumin (Fraction V, Nacalai Tesque Inc., Kyoto, Japan), 5% normal calf serum (Irvin Scientific, Kogan, UT, U.S.A.), 0.1% Tween 20 and 0.1% NaN₃. Blocking buffer-B consisted of PBS containing 1% bovine serum albumin, 5% normal calf serum, 0.1% Tween 20 and 0.01% thimerosal. GPU96MM, Sakai, IID5208 and C600 in logarithmic growth were washed with PBS containing 0.1% NaN₃ by centrifugation, and the bacterial cells suspended in PBS

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Table 1. *E. coli* Strains Used in This Study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Reference</th>
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<tbody>
<tr>
<td>GPU96MM</td>
<td>Clinical isolate in Gifu city (1996), Stx1 and Stx2-producing EHEC O157:H7</td>
<td>11), 17)</td>
</tr>
<tr>
<td>GPU991</td>
<td>espI-deletion mutant of GPU96MM</td>
<td>11)</td>
</tr>
<tr>
<td>GPU992</td>
<td>sepl-deletion mutant of GPU96MM</td>
<td>11)</td>
</tr>
<tr>
<td>Sakai</td>
<td>Clinical isolate in Sakai City (1996), Stx1 and Stx2-producing EHEC O157:H7</td>
<td>10)</td>
</tr>
<tr>
<td>A2-F6</td>
<td>Control mutant of Sakai by insertion of mini-Tn5Km2 into the 4769 bp site in Sakai-VT2 prophage, which is a pathogenicity-unrelated gene</td>
<td>10)</td>
</tr>
<tr>
<td>A6-E7</td>
<td>Mutant of Sakai by insertion of mini-Tn5Km2 into the sepl gene</td>
<td>10)</td>
</tr>
<tr>
<td>G1-E11</td>
<td>Mutant of Sakai by insertion of mini-Tn5Km2 into the tir gene</td>
<td>10)</td>
</tr>
<tr>
<td>Δeae</td>
<td>Nonpolar eae-deletion mutant of Sakai by replacing the portion containing the eae gene with an aphA-3 cassette</td>
<td>10), 38)</td>
</tr>
<tr>
<td>IID5208</td>
<td>Nonpathogenic <em>E. coli</em>, serotype O1:H7</td>
<td>10)</td>
</tr>
<tr>
<td>C600</td>
<td>K12, laboratory strain</td>
<td>10)</td>
</tr>
</tbody>
</table>
The results were expressed as the means±standard error of the mean (S.E.M.). 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 the non-parametric Duncan’s multiple range test (ranked multiple range test), to analyze the significance of differences between the 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 the non-parametric Duncan’s multiple range test (ranked multiple range test), to analyze the significance of differences between the groups. The significance of correlation coefficient (r) was analyzed by calculating the r-value. A value of p=0.05 was considered a significant difference in all statistical analyses.

RESULTS

Fecal Antigen-Specific IgA Antibodies Were Induced in Mice Inoculated with Wild-Type EHEC but Not with espA- and sepL-Deletion Mutants Mice were intragastrically inoculated once with $10^{11}$ CFU/kg of GPU96MM (wild type), GPU991 (espA-deletion mutant) and/or GPU992 (sepL-deletion mutant).

GPU96MM was detected at a stable level of $10^{5}$ to $10^{6}$ CFU/g feces from day 7 to day 28, while fecal shedding of GPU991 and GPU992 were quickly decreased and became undetectable by days 14 and 21, respectively (Fig. 1A).

The fecal IgA antibody titer in mice inoculated with GPU96MM began to rise at day 21 and then reached a plateau at day 28, whereas the titers in mice inoculated with GPU991 and GPU992 showed no increase (Fig. 1B).

Fecal Antigen-Specific IgA Antibodies Were Not Induced in Mice Even by 4 Repeated Inoculations of espA-Deletion Mutant, Heat-Killed EHEC and Nonpathogenic E. coli GPU96MM was intrastragically inoculated into mice once or 4 times at weekly intervals at a dose of $10^{11}$ CFU/kg. GPU991, heat-killed GPU96MM and nonpathogenic IID5208 were repeatedly inoculated 4 times at the same route and dosage schedule. Antibody titers against bacterial whole cells were examined for fecal and serum samples obtained 28 d after the first inoculation.

![Fig. 1. Fecal Shedding of Bacteria (A) and Fecal IgA Antibody Production in Mice Inoculated with GPU96MM (Wild Type), GPU991 (espA-Deletion Mutant) and GPU992 (sepL-Deletion Mutant) (B)](image-url)
In the group inoculated 4 times with GPU96MM, one mouse died on each of days 11, 15, 20, 23 and 26, respectively, and only 2 mice survived to day 28. Therefore, this group was excluded from the following analysis.

Mice inoculated once with GPU96MM (GPU96MM×1 mice) shed the bacteria into their feces at about $10^6$ CFU/g feces on days 3 and 6, and then the number of shed bacteria decreased gradually to day 28 (Fig. 2A). On the other hand, mice inoculated 4 times with GPU991 (GPU991×4 mice) shed the bacteria into their feces at 3 d after each inoculation but the shedding of bacteria decreased drastically at 6 d after each inoculation.

GPU96MM×1 mice showed a significantly high fecal IgA antibody titer against GPU96MM whole cells compared with normal mice on day 28 (Fig. 2B). The fecal IgA antibody titers in GPU991×4 mice and mice inoculated 4 times with heat-killed GPU96MM (killed GPU96MM×4 mice) were lower than that in GPU96MM×1 mice with statistical significance at $p\leq 0.01$. The titer against IID5208 whole cells in mice inoculated 4 times with IID5208 (IID5208×4 mice) increased slightly to a similar level to GPU991×4 and killed GPU96MM×4 mice. Serum IgM, IgG and IgA antibody ti-
tters showed an increase in all cases of bacterial inoculation, although the IgA antibody titers in GPU991 and killed GPU96MM were lower than that in GPU96MM mice with or without statistical significance (Fig. 2C). In the repeated experiment carried out using the same protocol, increases of the serum IgM, IgG and IgA antibody titers were reproducibly observed in all GPU991, killed GPU96MM and IID5208 mice (data not shown). The fecal IgA antibody titer did not show significant correlation with the serum IgA antibody titer in GPU96MM mice (Fig. 2D).

**sepL- and tir-Insertion Mutants Colonized in the Intestine of Km-Treated Mice for a Long Term, but Did Not Induce the Fecal Antigen-Specific IgA Antibodies**

Mice were given water containing 0.5 mg/mL Km throughout the experimental period, and inoculated intragastrically with $10^{10}$ CFU/kg of Km-resistant Sakai derivatives of A2-F6 having intact LEE, A6-E7 (sepL-insertion mutant) and G1-E11 (tir-insertion mutant) at 5 d after the start of the Km administration.

A2-F6 was shed into the feces at a higher level than A6-E7 and G1-E11 on days 3 and 6. However, the levels were nearly the same in all groups from day 14 to day 28 expect for that of A6-E7 on day 14 (Fig. 3A). In addition, fecal shedding of the bacteria even in A6-E7 were persistently detected higher than those in GPU96MM (Figs. 1A, 2A). Fecal IgA antibodies were detected on day 28 in mice inoculated with A2-F6, but not in mice inoculated with A6-E7 and G1-E11 (Fig. 3B). The serum IgM, IgG and IgA antibody titers showed an increase in mice inoculated with A2-F6, A6-E7 and G1-E11, although the serum IgA antibody titers in mice inoculated with A6-E7 and G1-E11 were significantly lower compared with that in mice inoculated with A2-F6 in contrast to the case of the serum IgM and IgG antibody titers (Fig. 3C).

**eae-Deletion Mutant Colonized in the Intestine of Km-Treated Mice for a Long Term, but Did Not Induce the Fecal Antigen-Specific IgA Antibodies**

Mice were given water containing 0.5 mg/mL Km throughout the experimental period, and inoculated intragastrically with $10^{10}$ CFU/kg of Km-resistant Sakai derivatives of A2-F6 and Δeae (eae-deletion mutation) at 5 d after the start of the Km administration.

Δeae continued to be shed into the feces until 28 d after the inoculation equally to or more than A2-F6 (Fig. 4A). Fecal IgA antibodies were detected in mice inoculated with A2-F6, but not in mice inoculated with Δeae at day 28 (Fig. 4B). Serum IgM, IgG and IgA antibodies were induced even in mice inoculated with Δeae, although the serum IgA antibody was rarely induced (Fig. 4C).

**DISCUSSION**

In the present paper, whole bacterial cells were used as the antigens to detect antibodies with filtration ELISA. Previously, we reported that colonies of GPU96MM were found on the epithelial surface of the ceca in association with F-actin accumulation beneath the attached bacteria following intragastrical inoculation into ICR mice, and fecal shedding of the bacteria was observed up to 3 weeks. In contrast, the adherence activities of GPU991 (espA-deletion mutant) and GPU992 (sepL-deletion mutant) to cultured HeLa cells were remarkably low compared with the parental wild-type strain GPU96MM, and fecal shedding periods of GPU991 and GPU992 were clearly shorter than that of GPU96MM. GPU96MM was shed into the feces continuously for 4 weeks following the single intragastrical inoculation and induced the fecal IgA antibody production in accordance with our previ-
Every group consisted of 7 mice. Data show the mean ± S.E.M. Statistically significant differences were found from the group of mice not inoculated with any bacteria (not-inoculated) (*) and from mice inoculated with A2-F6 (**) at p≤0.01.

Previously, we reported that the shedding of Δeae into feces decreased very quickly in contrast that the wild-type Sakai showed continuous shedding into feces of mice when inoculated intragastrically into mice that were not treated with antibiotics. It is well documented that treatment of mice with antibiotics to reduce intestinal micro-flora prolongs residing period of EHEC in the intestine. Actually, Km-treated mice showed prolonged and similar fecal shedding of Km-resistant Sakai-derived mutants of A2-F6, A6-E7, G1-E11 and Δeae. Here, A2-F6 having intact LEE increased the fecal IgA antibody titer but other mutants having defective LEE did not. Again, GPU991 (espA-deletion mutant), GPU992 (sepL-deletion mutant) and A6-E7 (sepL-insetion mutant) could not form the TTSS, and showed no adherence to cultured epithelial cells. On the other hand, G1-E11 (tir-insertion mutant) and Δeae (eae-deletion mutant) adhered diffusely to Caco-2 cells through the TTSS but not establish intimate adhesion through intimin–Tir interaction. Considering all together, the results strongly suggest that the adhesion of EHEC through the intimin–Tir interaction is essential for the induction of antigen-specific S-IgA antibody production in the intestine of mice. Some events such as signal transductions and/or cytokine productions in the host cells, which would occur after the binding of intimin and Tir, may play an important role in triggering the intestinal S-IgA antibody production.

It was reported that intimate adherence of EHEC is associated with intracellular signal transduction, such as the activation of protein kinase C and nuclear factor-κB (NF-κB), and an increase in intracellular Ca²⁺ in host cells. An increase in interleukin-8 production was observed in cells infected with EHEC O157:H7. It was indicated that adhesion of EHEC to host cells was essential for stimulating such signal transduction cascades. Thus, adhesion of EHEC to epithelial cells associated with the signal transduction may be a trigger to induce or a factor to potentiate the specific mucosal immune response on EHEC intestinal infection.

In contrast to the fecal IgA antibody titer, serum IgM and fecal antibody detected here was thought to be mainly that produced locally in the intestine and not transported from the blood through the bile, because there was no significant correlation found between the fecal IgA antibody titer and the serum IgA antibody titer in mice that received the single inoculation of GPU96MM (Fig. 2D). Local production in the intestine as a major source of the fecal IgA antibodies was also supported by the fact that the mice inoculated repeatedly with IID5208 showed a higher serum IgA antibody titer but a lower fecal IgA antibody titer.

The results discussed above suggested the importance of EHEC adherence to intestinal epithelial cells for induction of the mucosal IgA antibody. Then, it was examined which step of adherence contributed to the induction of IgA antibody in the following experiments using Sakai-derived mutants of A6-E7, G1-E11 and Δeae. Both GPU96MM and Sakai are clinical isolates (serotype O157:H7, producing Stx-1 and Stx-2) obtained from patients in the outbreaks occurred in June and July 1996 in the cities of Gifu and Sakai, Japan, respectively. There is no information about difference between GPU96MM and Sakai except that Izumiya et al. classified GPU96MM into Ib and Sakai into Iia by pulsed-field gel electrophoresis patterns.

There is no information about difference between GPU96MM and Sakai except that Izumiya et al. classified GPU96MM into Ib and Sakai into Iia by pulsed-field gel electrophoresis patterns. 23) Previously, we reported that the shedding of Δeae into feces decreased very quickly in contrast that the wild-type Sakai showed continuous shedding into feces of mice when inoculated intragastrically into mice that were not treated with antibiotics. 24) It is well documented that treatment of mice with antibiotics to reduce intestinal micro-flora prolongs residing period of EHEC in the intestine. 25,26) Actually, Km-treated mice showed prolonged and similar fecal shedding of Km-resistant Sakai-derived mutants of A2-F6, A6-E7, G1-E11 and Δeae. Here, A2-F6 having intact LEE increased the fecal IgA antibody titer but other mutants having defective LEE did not. Again, GPU991 (espA-deletion mutant), GPU992 (sepL-deletion mutant) and A6-E7 (sepL-insetion mutant) could not form the TTSS, and showed no adherence to cultured epithelial cells. 19,18) On the other hand, G1-E11 (tir-insertion mutant) and Δeae (eae-deletion mutant) adhered diffusely to Caco-2 cells through the TTSS but not establish intimate adhesion through intimin–Tir interaction. 20) Considering all together, the results strongly suggest that the adhesion of EHEC through the intimin–Tir interaction is essential for the induction of antigen-specific S-IgA antibody production in the intestine of mice. Some events such as signal transductions and/or cytokine productions in the host cells, which would occur after the binding of intimin and Tir, may play an important role in triggering the intestinal S-IgA antibody production.

It was reported that intimate adherence of EHEC is associated with intracellular signal transduction, such as the activation of protein kinase C and nuclear factor-κB (NF-κB), 27,28) and an increase in intracellular Ca²⁺ in host cells. 27) An increase in interleukin-8 production was observed in cells infected with EHEC O157:H7. 27) Ismaili et al. indicated that adhesion of EHEC to host cells was essential for stimulating such signal transduction cascades. 29,30) Thus, adhesion of EHEC to epithelial cells associated with the signal transduction may be a trigger to induce or a factor to potentiate the specific mucosal immune response on EHEC intestinal infection.

In contrast to the fecal IgA antibody titer, serum IgM and
IgG antibody productions as a systemic immune response were induced even in the adhesion-deficient mutants. However, the mutants, especially Δ*eae* (Fig. 4C) showed a tendency of lower serum antibody titers, suggesting that adherence of EHEC to the intestinal mucosa facilitates the systemic immune response although it is not essential. The mechanism for the induction of the systemic immune response against the intestinal antigens is not precisely known. It has been reported for the induction of the systemic immune response against the EHEC to the intestinal mucosa facilitates the systemic immune response through intimin–Tir interaction as an intimate adhesion, not only through TTSS as an initial attachment but also through intimin–Tir interaction as an intimate adhesion, although TTSS is not sufficient for induction of the systemic immune response.

With regard to the serum IgA antibody production, the results were complex compared with those for serum IgM and IgG antibody productions. The inoculation of EHEC with intact adhesive activity (wild type of GPU96MM and A2-F6) induced serum IgA antibody production as well as the fecal IgA antibodies. On the other hand, there was a difference in the serum IgA antibody productions between single and repeated inoculations of the adhesion-deficient bacteria. The repeated inoculation of GPU991, heat-killed GPU96MM and IID5208 induced serum IgA antibody production, but single inoculations of the 3 adhesion factor-deficient mutants, A6-E7, G1-E11 and Δ*eae*, did not. This difference may result from differences in the state of the bacterial antigens in the intestine between single and repeated inoculations. Previously, we reported that GPU96MM colonized mainly in the cecum but not in the small intestine of mice not treated with Km. In the case of the single intragastrical inoculation of the mutants into Km-treated mice, the mutants also mainly detected in the cecum (data not shown). On the other hand, the bacteria that were inoculated repeatedly passed through the bactericidal stomach at each of the 4 inoculations, and antigens released from the killed bacteria might stimulate serum IgA antibody production. In conclusion, it is essential for the induction of the mucosal immune response that EHEC adheres to intestinal mucosa not only through TTSS as an initial attachment but also through intimin–Tir interaction as an intimate adhesion, but not for induction of the systemic immune response.

The cause of death of mice inoculated repeatedly 4 times with GPU96MM might be Stxs produced from the bacteria. However, diarrhea was not observed in any mice of any inoculation even 4 times of GPU96MM. Previously, we reported that neither diarrhea nor fecal occult blood was observed in mice following the single inoculation of GPU96MM. In addition, histopathological changes such as tissue swelling, bleeding or inflammatory cell infiltration were not observed by right microscopic examination of sections stained with hematoxylin and eosin in any part of the intestine when examined until 28 d after the GPU96MM inoculation.

Further study will be required to clarify how and what intracellular signal transductions and/or cytokine productions following the adhesion trigger the antigen-specific S-IgA antibody production and/or isotype switching to IgA-producing B cells in the intestine. It also remains to be studied in the future whether the adhesion plays some role in homing the antigen-specific IgA antibody-producing B cells to the lamina propria of the intestine.

REFERENCES


