Gastrointestinal Antibody Responses in Axenic Mice to Topically Administered Escherichia coli

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Immunoglobulin levels were determined in gastrointestinal secretions of both conventional and axenic mice, as was the stability of the immunoglobulin classes in these secretions. Axenic animals were then administered nonviable Escherichia coli O111:B4 by topical application into the oral cavity. Immunoglobulin (Ig) A, IgG1, and IgG2 were detected in the gastrointestinal secretions, with an apparent gradation in stability (IgA > IgG1 > IgG2) under the conditions investigated. Specific antibodies (IgA and IgG) to both the somatic and the flagellar antigens of E. coli were demonstrated. The time course of the responses to the polysaccharide and protein antigens suggested that a secondary response to lipopolysaccharide was being observed in the secretions. Examination of the autoclaved diet fed to the germfree animals indicated the presence of antigenically intact polysaccharide materials (i.e., lipopolysaccharides), whereas antigenic bacterial proteins were uniformly undetectable. Therefore, endotoxin in the diet was apparently causing a primary type of sensitization in the mice, whereas the active oral immunization regimen induced a secondary antibacterial response in the secretions.

Immune factors altering the replication or activity of intestinal microorganisms may be important in controlling the normal intestinal microflora (6, 43, 46). Such a system could play a significant role in host resistance to intestinal infections. Gastrointestinal (GI) secretions have been shown to contain antibody activity against a variety of viral and bacterial agents after immunization or natural infection (2, 12, 13, 15, 33, 34, 47). The normal intestinal mucosa has been shown to contain a large number of plasma cells and is considered to be an active component of the mammalian lymphoid system (6, 9, 16, 20, 43). It has likewise been demonstrated that the plasma cells inhabiting the lamina propria of the intestine are primarily of the immunoglobulin A (IgA) type (6-8, 38). The predominance of IgA-staining plasma cells in the GI tract is reflected in the substantial level of IgA present in the GI secretions (1, 4, 6, 28, 36). Nash et al. (31) and Benveniste et al. (1) showed that the IgA found in mouse intestinal secretions was primarily of the 11S type, which is characteristic of exocrine secretory IgA. Similar results demonstrated that the predominant immunoglobulin class against both bacteria and viruses in this biological secretion was secretory IgA (2, 14, 21, 23, 34, 38). It was also suggested that the mode of immunization is the primary determinant in the production of secretory antibodies (24, 25, 34). This study was therefore undertaken to determine whether topical administration of nonviable Escherichia coli was capable of eliciting a GI humoral immune response in axenic mice. The kinetics, specificity, and classes of antibodies produced in the intestinal secretions after peroral sensitization were also examined.

MATERIALS AND METHODS

Experimental protocol. Conventional and axenic ICR/CD-1 mice were used in the study when 2 to 4 months old. The preparation of E. coli O111:B4 antigens for immunization and analysis of antibody production has been described previously (11). Forty-eight axenic mice were immunized by daily oral swabbing with a suspension of Formalin-killed E. coli. Cotton swabs used in the immunization procedure contained approximately 0.25 ml of the antigen preparation (5 × 10⁶ organisms). Groups of mice were removed (six mice per group) from the germ-free isolators after each swabbing on days 1, 3, 5, 7, 9, 11, 13, and 15. GI perfusates were collected from the individual animals 24 h after the final antigen administration. Control GI secretions were obtained from untreated mice or from animals that had been orally swabbed with sterile, pyrogen-free saline (Abbott Laboratories, North Chicago, III.) for a similar 14-day regimen.

Collection of specimens. Serum and pilocarpine-stimulated saliva samples were collected and prepared as described previously (30).
The GI perfusates were obtained from conventional and axenic mice that had been used for collection of saliva and serum. The animals were exsanguinated under ether anesthesia, and the entire small intestine, from the gastric sphincter to the cecum, was excised. The intestine from each mouse was subsequently cut into 1-cm sections and suspended in 5 ml of chilled 0.15 M NaCl. This suspension was mixed vigorously for 10 min, followed by centrifugation at 2,000 × g for 15 min. The cloudy supernatant was then removed and frozen at −20°C overnight. After thawing, the suspension was clarified by recentrifugation at 4,000 × g for 20 min. The perfusate samples were then heated at 56°C for 30 min to minimize proteolytic activity (36), and Merthiolate was added (0.1%) to the samples to inhibit any residual bacterial growth. Protein determinations on numerous individual samples (27) have indicated that the protein content of the perfusates was approximately 15 mg/ml. Single radial immunodiffusion analyses of the samples were performed according to Molinari et al. (30) and demonstrated immunoglobulin levels depicted in Table 1.

Preparation of homogenate from axenic mouse diet. The axenic mice were fed an autoclaved GBI TDD70061 diet (General Biochemicals Div. [Mogul Corp.], Chagrin Falls, Ohio) and water ad libitum. A 300-g amount of the autoclaved pelleted diet was suspended in 400 ml of sterile 0.15 M NaCl. This mixture was placed in a sterile Waring blender and minced at the high-speed setting for 25 min. The suspension was subsequently centrifuged at 2,000 × g of nitrogen-containing protein per ml. The antigen tics, Oxnard, Calif.). The resulting supernatant was removed, concentrated to approximately 10 ml by rapid pervaporation, and used as the sensitizing antigen in a passive hemagglutination (PA) procedure. A protein determination was performed (27) to standardize the preparation for use in the serological assay, and the sample was found to contain 1.365 g/ml of nitrogen-containing protein. The antigen preparation was diluted to 50 mg/ml and used to coat sheep erythrocytes (SRBC) after treatment with tannic acid.

Antibody analyses. Reciprocal antibody titers in the GI secretions were determined by PA, using antigen-coated SRBC (30). The class(es) of specific agglutinins was demonstrated by adsorption of the secretions with monospecific antisera (IgA and IgG) and by 2-mercaptoethanol sensitivity (IgM) (11). Statistical analyses of the data were performed using Student's t test applied to the arithmetic means of the reciprocal antibody titers.

RESULTS

Stability of immunoglobulins in intestinal perfusates. Degradation of immunoglobulins in the intestinal tract, presumably by proteolytic enzymes, has been suggested by various studies of the alimentary local immune response (13, 32, 36). A modification of the technique of Plaut and Koenil (36) was used to stabilize the immunoglobulin content of GI perfusates obtained from conventional and axenic mice. The perfusates were heat treated according to the original procedure, and Merthiolate was added to the samples. The specimens were subsequently frozen at −20°C until assayed for antibody activity. To determine the effect of heat and Merthiolate treatments on the stability of the immunoglobulins in the GI secretions, portions from random samples of the perfusates were incubated at 4 and 25°C for an interval of up to 22 days. Similar untreated specimens were incubated under identical conditions. The concentrations of IgGl, IgG2, and IgA were measured by the radial immunodiffusion technique at 3, 7, 11, 15, and 22 days after obtaining the intestinal perfusates. The results are depicted in Fig. 1. Samples incubated at 4°C after treatment to abrogate proteolytic activity by enzymes and bacterial growth retained approximately 64, 56, and 70% (IgGl, IgG2, and IgA, respectively) of their initial concentrations of immunoglobulins. Treatment of the specimens prior to incubation at 25°C was also able to inhibit breakdown of the immunoglobulins. At 22 days, 28% (IgGl) and 8% (IgG2) of the initial concentrations of these immunoglobulins were still present in the perfusates. IgA appeared to exhibit a greater stability under these conditions, with 34% of the original level present after 22 days. Analysis of samples of the untreated specimens suggested a difference in stability of immunoglobulin classes in the intestinal secretions. Incubation at 4°C resulted in only 12% of the initial IgG2 concentration still detectable by day 7, whereas at 11 days this immunoglobulin class was absent from the specimens. The level of IgGl was 14% at 11 days and disappeared by day 15. Similarly, detectable levels of IgA were absent at day 15; however, 22% of the initial concentration of this immunoglobulin was present at 11 days. When the specimens were incubated at 25°C with no prior treatment, no IgG was detectable by 3 days, whereas IgA activity did not totally disappear until day 7. Consequently, treatment of the perfusate samples with heat and Merthiolate

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Immunoglobulin concn*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgGl</td>
</tr>
<tr>
<td>Conventional</td>
<td>59 ± 7</td>
</tr>
<tr>
<td>Axenic</td>
<td>21 ± 3</td>
</tr>
</tbody>
</table>

* Immunoglobulin concentration is reported as micrograms per milligram of protein in the perfusate samples, mean level of immunoglobulin with range for six samples.  
  b—, No IgM was detected.
appeared to significantly increase the stability of the various immunoglobulins in these secretions.

The catabolism of IgA was also determined in perfusates from axenic mice. Various reports have indicated that axenic animals have increased concentrations of intestinal enzymes (20, 28, 40, 49), which could result in an enhanced breakdown of immunoglobulins in the alimentary canal. The results shown in Fig. 1 tend to substantiate an increased enzymatic degradation of immunoglobulins in the intestinal tract of axenic mice, when compared to their conventional counterparts. Untreated perfusate specimens from the germfree mice were devoid of IgA by 7 days, after incubation at either 4 or 25°C. In contrast, treatment of the samples with heat and Merthiolate abrogated a substantial portion of the catabolism taking place at either temperature. A difference between the two groups was also noted for those samples treated and incubated at 25°C. Conventional mouse perfusates at 22 days exhibited a significantly lower percentage \( (P < 0.05) \) of the initial IgA level (34%) when compared to the percent concentration in specimens from the axenic animals (54%). This could presumably be due to the difference in levels of bacterial products following treatment of the holoxenic and axenic secretions.

Response to *E. coli* somatic (O) antigen. The local immune response in the GI tract of axenic mice was examined by both PA and bacterial agglutination (BA). Specific anti-lipopolysaccharide (LPS) antibodies were detected in the GI perfusates of immunized mice within 24 h after initiation of the regimen (Fig. 2). Antibody levels attained peak titers by day 5 and remained at this level throughout the immunization interval. Similar results were demonstrated with BA as the serological assay. By 24 h, six of six mice exhibited anti-O agglutinins in the GI washings (Fig. 3). Antibody titers peaked at day 7 and leveled off for the remaining 8 days of oral sensitization.

The classes of antibodies in the GI perfusates of orally sensitized axenic mice, induced by the somatic antigen of *E. coli*, paralleled those reported for other exocrine secretions (10, 11, 30). The early antibody produced in the GI tract was IgG; however, IgA agglutinins increased significantly during the immunization regimen. From approximately 9 to 11 days until the end of the sensitization period, IgA comprised 50 to 75% of the antibody detected in the perfusates by PA (Fig. 2) and BA (Fig. 3). Treatment of the perfusates with 2-mercaptoethanol had no effect on the antibody titers, suggesting a lack of IgM agglutinins in these samples.

Response to *E. coli* flagellar (H) antigen. GI

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![Graphs showing stability of immunoglobulins in GI perfusates from conventional (CVM) and germfree (GFM) mice.](http://iai.asm.org/)
GASTROINTESTINAL ANTIBODY RESPONSES TO E. COLI

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Perfusates of axenic mice during oral sensitization with E. coli, as determined by PA. Symbols: (O) Mean total reciprocal antibody titer, with range of values for six mice indicated by vertical bar (()). Bars indicate mean titer of antibody activity remaining after removal of IgG by prior treatment with anti-IgG serum. Selected samples were also absorbed with anti-IgA serum, which removed the remainder of the agglutinin activity.

Perfusates were assayed in a similar manner for antiflagellar antibodies throughout the immunization regimen. The production of agglutinins against the E. coli flagellar antigen differed from that observed with the anti-O antibodies. Specific agglutinins were detectable in all perfusate samples at 5 days after initiation of the sensitization regimen; however, antibodies were demonstrated in some samples as early as 3 days. Antibody titers peaked at 11 days and remained at a mean reciprocal titer of 32 until the end of the assay period (Fig. 4).

Absorption studies were used to determine the class(es) of antiflagellar antibodies detected in the GI secretions. IgG represented the initial antibodies elicited by peroral administration of the nonviable E. coli. However, by day 9 of the sensitization procedure, IgA was the predominant immunoglobulin present in this exocrine secretion and comprised 60 to 75% of the agglutinating activity detected throughout the remaining interval of sensitization (Fig. 4).

Specificity of the GI antibody response. Heterologous antigen preparations were used to examine the specificity of the antibody produced in response to oral sensitization. The perfusate samples were tested by PA against Salmonella typhi group D and Salmonella paratyphi-B group B somatic antigens. Also, a PA technique, using the carbohydrate antigen of group H streptococci (Difco Laboratories, Detroit, Mich.), was used in assessing antibody specificity. The GI perfusates from axenic mice administered nonviable E. coli showed a threefold rise in perfusate anti-LPS agglutinins, with no concomitant increase in agglutinating capacity against the heterologous antigens (Table 2).

To demonstrate that the antibodies detected with the flagellar preparation were directed exclusively towards the H-determinant, the samples were reacted against the E. coli O111 somatic antigen used in the BA assays. After incubation at 37°C for 1 h and 4°C overnight, the bacteria were pelleted by centrifugation at 1,400 x g. The resulting supernatant was subsequently used in an agglutination assay against the antigen preparations. None of the antiflagellar antibodies (day 15) were removed by this procedure, whereas this absorption abrogated the agglutination of E. coli O111 and E. coli O111:B4 serological antigens, as well as SRBC sensitized with E. coli LPS (Table 2).
Duplicate samples of the perfusates were also reacted against the H antigen of S. typhi in an agglutination assay. The results demonstrated that axenic mice were capable of exhibiting a specific local exocrine immune response directed toward both somatic and flagellar antigenic determinants of the orally administered E. coli.

Immune response of axenic mice to antigens in the germfree diet. In the process of examining the GI perfusates, it was noted that the latent period until detection of anti-LPS antibodies was either of very short duration or nonexistent in some samples. Consequently, it was felt that the axenic diet fed to the mice may contain the heat-stable endotoxin of certain gram-negative bacteria. Therefore, the mice would actually be receiving a constant antigenic challenge; however, since antibodies were undetected in germfree mice that had not been orally swabbed with the microorganisms, the antigen must be present in a level below that required for the induction of exocrine antibodies. To investigate this possibility, a homoge-
nate was prepared from the autoclaved diet and
attached to SRBC with tannic acid. Specific
antisera were then reacted against the sensi-
tized cells to determine the types of bacterial
antigens that may be present in the autoclaved
diet. Protein antigens appeared to be absent
from the sterilized pellets, whereas polysac-
charides remained antigenically intact (Table
3). Table 3 also shows that serum and secre-
tions from orally immunized mice agglutinated
the sensitized SRBC. To determine the speci-
city of this reaction for E. coli LPS, selected
samples were absorbed with the E. coli O111
somatic antigen, and the resulting superna-
tants were reassayed for agglutinin capabil-
ities. After removal of anti-E. coli O antibodies,
there was a substantial decrease in agglutina-
tion of the sensitized SRBC (Table 3). Simi-
larly, during local immunization of axenic mice
with Formalin-treated E. coli, the reciprocal
titers of antibodies capable of agglutinating
SRBC sensitized with the diet homogenate in-
creased in the exocrine secretions (salivary and
GI (Fig. 5). These results suggested the pres-
ence of E. coli antigens in the pelleted diet after
autoclaving.

DISCUSSION
The digestive mucosal surface has been de-
scribed as an important site of interaction of an
organism with its surroundings. From an im-

<table>
<thead>
<tr>
<th>Group†</th>
<th>Sample</th>
<th>Treatment‡</th>
<th>Agglutinins to: SRBC coated with homogenate of mouse diet</th>
<th>Tannic acid-treated SRBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVM control‡</td>
<td>Saliva</td>
<td>Serum</td>
<td>14 (4-16)§</td>
<td>&lt;2'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GI perfusate</td>
<td>12 (4-16)</td>
<td>&lt;2</td>
</tr>
<tr>
<td>CVM immunized with E. coli</td>
<td>Saliva</td>
<td>Serum</td>
<td>68 (16-128)</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GI perfusate</td>
<td>5 (&lt;2-8)</td>
<td>&lt;2</td>
</tr>
<tr>
<td>GFM control</td>
<td>Saliva</td>
<td>Serum</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GI perfusate</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>GFM immunized with E. coli</td>
<td>Saliva</td>
<td>E. coli O111</td>
<td>8 (4-16)</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>Saliva</td>
<td>E. coli H antigen</td>
<td>3 (&lt;2-8)</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>Saliva</td>
<td>E. coli O111</td>
<td>8 (4-16)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>E. coli O111</td>
<td>3 (&lt;2-8)</td>
<td>ND</td>
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<td></td>
<td>GI perfusate</td>
<td>8 (4-16)</td>
<td>&lt;2</td>
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<td></td>
<td>GI perfusate</td>
<td>E. coli O111</td>
<td>3 (&lt;2-8)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>GI perfusate</td>
<td>E. coli H antigen</td>
<td>8 (4-16)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Antiserum

| E. coli O111 | 6‡ | <2 |
| E. coli O111:B4 | 16 | <2 |
| Salmonella O antigen group D | 8 | <2 |
| Salmonella O antigen group B | 16 | <2 |
| Salmonella H antigen d | <2 | <2 |
| Salmonella H antigen i | <2 | <2 |
| Streptococcus group H | 4 | <2 |
| Streptococcus group D | <2 | <2 |
| Salmonella Vi antigen | <2 | <2 |

† CVM, Conventional mice; GFM, germfree mice.
‡ Indicates absorption of samples prior to agglutinin assay.
§ Tannic acid was used to treat SRBC to enhance attachment of soluble substances in the homogenate.
¶ Control mice were swabbed with sterile, pyrogen-free physiological saline.
* Mean reciprocal antibody titer, with range for six mice indicated in parentheses.
• No antibodies detected by this serological assay.
* ND, Not done.
‡ Mean of triplicate determinations of a single sample of antiserum.
munological standpoint, this contact is reflected in the mucosa by well-organized lymphoid tissue, such as lymphoid follicles and free plasma cells. In contrast, the alimentary tract of axenic mice contains little lymphoid tissue. Plasma cells are very sparse in the lamina propria, and the lymphoid follicles are absent or poorly developed (6, 9, 16, 18, 22, 42).

McClelland et al. (29) demonstrated that IgA in the GI secretions was capable of agglutinating a wide range of enteric microorganisms. Similar results have shown that the predominant immunoglobulin class of antibodies to pathogenic bacteria in these biological secretions from preruminant calves (39), sheep (35), young pigs (37), dogs (44), and mice (1) was secretory IgA. Consequently, secretory antibodies in the GI secretions appear to be important in determining the normal flora of the alimentary tract, as well as exhibiting a protective capacity against enteric pathogens.

Recently, this laboratory has investigated the kinetics and specificity of the local immune response in the oral cavity after topical administration of bacterial immunogens (10, 11, 30). As a result of these studies, the mouse appeared to offer a model for determining the immunological reactions of an organism to its local septic environment. By using axenic mice in this investigation, a detailed examination of the secretory immune system in the GI tract of this mammal was permitted. This study has examined the characteristics of the intestinal immune system in germfree mice after a similar topical immunization regimen.

Peroral sensitization of axenic mice with nonviable E. coli induced a specific GI immune response to the somatic antigen of this microorganism. In examining the kinetics of this reaction, some of the animals exhibited antibodies within 24 h after initiation of the regimen, whereas antibody titers were observed to peak by days 9 to 11 and to remain at this level throughout the study. The classes of immunoglobulins produced in the intestinal perfusates in response to the peroral sensitization were found to be similar to those observed in the salivary secretions of these animals (11). The initial immune response to both the somatic and flagellar antigens was found to be IgG; however, by 9 to 11 days IgA was the predominant antibody class in this secretion. An interesting observation was obtained from the PA serological technique, in that specific IgA anti-LPS antibodies were demonstrated in the perfusates of some animals within 24 h of the initial sensitization. These results could be expected because of detectable levels of IgA (antibodies) in the axenic mouse secretions prior to oral immunization (1; see Table 1). These results suggested that the mice may have been receiving prior antigenic stimulation with LPS, although no anti-E. coli antibodies were detected in the saliva and serum (11) or GI perfusates before initiation of active immunization. In contrast, various studies have demonstrated the presence of antibodies in axenic mouse serum to certain bacteria, including E. coli (5, 19, 41, 45). The sensitivities of the serological analyses used in these studies appear to account for
the ability to detect low amounts of antibodies present in the germfree specimens. Therefore, due to reports that germfree animal diets are not devoid of antigenic materials, which appear to be the agents that elicit these serum antibodies (17, 26, 48), the sterilized diet used in this investigation was examined for \textit{E. coli} antigens.

The homogenate from the diet was shown to react with saliva, serum, and GI perfusates from immunized axenic mice. This agglutination capacity was blocked by treatment of the samples with the \textit{E. coli} somatic antigen. Similarly, serological tests with specific antisera indicated that there was a variety of bacterial antigens present in the diet composed almost exclusively of polysaccharide substances. Antisera to various protein antigens suggested a lack of these materials following autoclaving of the pelleted feed. Subsequently, it appeared that the germfree mice were receiving a constant challenge with the \textit{E. coli} O antigen; however, the quantities were below a level required for induction of exocrine antibodies. After initiation of the orally administered \textit{E. coli}, antibodies were immediately detected at the local intestinal site. These results concur with data reported by Buscho et al. (3), whereby human volunteers were challenged locally with a dose of rhinovirus that would normally be nonimmunogenic. However, those individuals who had a previous infection with a homologous serotype of the virus exhibited a substantial local antibody response. Consequently, these reactivities may be considered a type of secondary immune response, where an immunological memory is functioning at the local site of antigen administration. In contrast, the intestinal response to the proteinaceous flagellar antigen differed from that observed with antisomatic antibody production. The peak reciprocal titers to the O and H antigens were similar in the GI secretions of the axenic mice; however, the latent interval before antibody detection was substantially longer for antiflagellar antibodies (~5 days) when compared with antisomatic antibody production (~1 day). These results could be attributed to the lack of antigenic proteins (i.e., flagella) in the sterilized feed, whereas gram-negative bacterial endotoxins were present in the diet. Therefore, the local response to the flagella of \textit{E. coli} appears to be a true primary immune reaction in the GI tract of the germfree mice.

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