Immune-Mediated Emigration of Neutrophils into the Lumen of the Small Intestine

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By using pigs sensitized to bovine serum albumin (BSA), it was found that exposure of the intestinal mucosa to BSA induced, in 4 h, the emigration of large numbers of neutrophils into the intestinal lumen. This response was specific for the immunizing antigen and could be transferred to nonimmune animals with immune serum. The emigration of neutrophils through the intestinal mucosa was not accompanied by the edema, hemorrhage, and thrombosis which were apparent after intracutaneous inoculation of BSA into the same animals. Twenty-four hours after a 4-h mucosal exposure to BSA, the intestinal mucosa showed no evidence of neutrophil emigration nor any other abnormal features. These observations suggested that emigration of neutrophils into the intestinal lumen can be a specific, antibody-mediated immune response which occurs in the absence of intestinal injury. Possible relationships between immune-mediated enteroluminal emigration of neutrophils, neutrophil production, and a protective role for the neutrophil in the intestinal lumen were considered.

How local immune mechanisms protect the intestinal mucosa from action or invasion by pathogenic bacteria remains uncertain. Williams and Gibbons (19) demonstrated that secretory immunoglobulin (Ig) A specifically inhibits the adherence of bacteria to epithelial cells and suggested that local antibody may be protective by preventing bacterial colonization of mucosal surfaces. Intestinal antibodies might also act as opsonins. Although reports on the opsonic capabilities of secretory IgA are contradictory (3, 6, 9, 21), antibodies of the IgG or IgM class in intestinal secretions could also exert this function.

Experimental evidence suggests that the intestinal tract is normally the principal site of granulocyte elimination (18). Also, proliferation of intestinal coliform bacteria has been shown to increase neutrophil emigration into the intestinal lumen (C. W. Yong, M.Sc. Thesis, Univ. of Saskatchewan Saskatoon, 1971). If phagocytosis is an effector of the intestinal immune response, it seems likely that a tropic mechanism would exist to attract phagocytes into the intestinal lumen. We have therefore examined the possibility that emigration of neutrophils into the intestinal lumen could be a specific, immunologically mediated response.

MATERIALS AND METHODS

Weanling pigs weighing 8 to 10 kg were used in all experiments.

Immunization. Animals were actively immunized to bovine serum albumin (BSA) with three subcutaneous inoculations of 140 mg of BSA (Nutritional Biochemicals, Cleveland, Ohio) incorporated in complete Freund adjuvant (Difco Laboratories, Detroit, Mich.). A nonimmune control group received complete Freund adjuvant alone. Animals were passively immunized by administering anti-BSA serum (8.0 ml/kg) intravenously. (The donors of anti-BSA serum were immunized as described above and elicited an Arthus-like reaction after intradermal inoculation of BSA). The nonimmune control groups received serum from animals immunized to Freund adjuvant alone.

Intestinal loop and skin testing. After an 18-h fast, pigs were anesthetized and the right lateral abdominal wall was incised. Eight 10-cm loops, separated by 8-cm interloops, were isolated by silk ligatures. Beginning in the anterior jejunum, approximately 100 cm distal to the pylorus, ligation was done carefully to preserve the blood supply to each loop. When ligation was completed, the test materials were injected into the lumen of each loop with a 23-gauge needle, taking care to avoid the puncture of blood vessels. The following eight test materials were injected into the ligated intestinal loops: BSA, BSA plus anti-BSA, BSA plus anti-BSA plus complement, BSA plus complement, BSA plus heated normal pig serum (HNS), complement alone, HNS alone, and mannitol alone. BSA was inoculated at a concentration of 525 mg per 10-cm loop. Two milliliters of

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pooled hyperimmune serum from pigs immunized as above and heated to 56°C for 30 min served as anti-BSA. Normal fresh pig serum (0.5 ml) was used as the source of complement, and 2.0 ml of the same serum, heated to 56°C for 30 min, was used as the HNS. Each inoculum was made up to 6.0 ml with slightly hypertonic (325 mosmol/liter) mannitol, a nonabsorbable sugar, to provide an adequate volume of intestinal loop fluid for cytological studies. Precipitation tests indicated that the inocula containing both BSA and anti-BSA were in great antigen excess. BSA, BSA plus anti-BSA, and mannitol were also inoculated intradermally for comparison of cutaneous and intestinal responses. Four hours after loop inoculation, the length of each loop was measured, the volume of loop fluid was recorded, and pieces of intestine were taken for histopathological examination.

Cytological and histopathological methods. Two smears of each loop fluid were dried, fixed, and stained; one was prepared with peroxidase reagent by the method of Rytömäa (16) and counterstained with 1% toluidine blue, and the other was prepared with Wright-Giemsa stain. The peroxidase reagent rendered neutrophil and eosinophil granules a dark-black color so that they were easily differentiated from the blue-staining epithelial and other mononuclear cells. A comparison between peroxidase-stained smears and Wright-Giemsa-stained smears indicated that when a test loop had an increase in peroxidase-positive cells, the increase was due entirely to an influx of neutrophils. Also, it was observed on histological sections of intestine that the number of neutrophils migrating through the mucosa of a particular loop varied from villus to villus but the mean number of neutrophils in 30 or more villi was nearly constant. For these two reasons, it was assumed that the eosinophil numbers would be negligible and the total number of neutrophils migrating through the epithelial surface per centimeter of intestine would be constant throughout a given loop and could be computed as:

Neutrophils/cm = \( \frac{(\text{total cells}/\text{loop fluid [ml]}) \times (\text{total volume of loop fluid [ml]})}{(\text{neutrophils [%]})/(\text{loop length [cm]})} \)

Total cell counts were determined on an autocytometer (Fisher Scientific Co., Pittsburgh, Pa.).

For histological examination, pieces of intestine were either fixed in Bouin-Holland fluid or frozen at \(-20°C\) in preparation for frozen sectioning. The skin injection sites were bisected; one-half was fixed in 10% neutral buffered Formalin, and the other was frozen at \(-20°C\) for frozen sectioning. The Bouin-Holland- and Formalin-fixed tissues were dehydrated, paraffin embedded, sectioned at 6 \(\mu\m\), and stained with hematoxylin and eosin. Frozen sections of intestine and skin were cut at approximately 8 \(\mu\m\). The frozen sections were fixed in Formalin-alcohol solution, stained by the peroxidase method of Rytömäa (16), and counterstained with 1% toluidine blue.

The assessment of histopathological lesions in all intestinal loops and skin inoculation sites was based on their deviation from the histological appearance of the control (inoculated with mannitol). A double-blind method was used for assessment of histological lesions.

Statistical methods. The loop-testing materials were randomized before inoculation. The data for total cell counts were subjected to analysis of variance, and the means for each treatment and each group were compared by using Duncan’s multiple-range tests (10).

RESULTS

The magnitude of enteroluminal neutrophil emigration in response to the various inocula is shown in Table 1. After mucosal exposure to BSA, the immunized group showed a 20- to 30-fold increase in the numbers of neutrophils entering the intestinal lumen when compared with the nonimmune group. No additional increase in neutrophil numbers resulted from the presence of anti-BSA, complement, or HNS with BSA. In the nonimmune pigs, neutrophil numbers did not vary significantly with the different inocula. Of particular note was the lack of intestinal neutrophil response to BSA plus anti-BSA in the nonimmune group. This contrasted with the Arthus-like reaction observed after intracutaneous inoculation of the identical material into the same animals.

Histological examination of intestinal loops in immune animals revealed only one major difference from nonimmune animals after mucosal exposure to BSA. Neutrophils accumulated focally in the epithelial layer and the immediate subepithelial capillaries (Fig. 1). Other signs of

<table>
<thead>
<tr>
<th>Loop inocula</th>
<th>No. of neutrophils ((x 10^6/cm) of intestine)</th>
</tr>
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<tbody>
<tr>
<td>BSA</td>
<td>6.6*</td>
</tr>
<tr>
<td>BSA + anti-BSA</td>
<td>5.4*</td>
</tr>
<tr>
<td>BSA + anti-BSA + C</td>
<td>6.2*</td>
</tr>
<tr>
<td>BSA + C</td>
<td>6.4*</td>
</tr>
<tr>
<td>BSA + HNS</td>
<td>4.9*</td>
</tr>
<tr>
<td>C</td>
<td>0.2</td>
</tr>
<tr>
<td>HNS</td>
<td>0.4</td>
</tr>
<tr>
<td>Mannitol</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* Determinations were made 4 h after mucosal exposure to BSA and control inocula. Values represent the mean of six pigs.

\(^*\) BSA, C, and HNS represent bovine serum albumin, complement, and heated normal pig serum, respectively.

\(^*\) Significantly different (\(P < 0.01\)) from mannitol control.
inflammation such as edema, hemorrhage, and thrombosis were not observed. Inoculation of complement, HNS, or mannitol into ligated loops of BSA-immune animals elicited no response. In the nonimmune group, the mucosa of every intestinal loop appeared normal (Fig. 2). Intradermal inoculation of BSA into immune animals induced an Arthus-like inflammatory reaction consisting of edema, hemorrhage, and thrombosis, together with perivascular accumulations of neutrophils. In the nonimmune group, intradermal BSA and mannitol elicited a mild, diffuse infiltration of neutrophils without edema, hemorrhage, or thrombosis, whereas BSA plus anti-BSA induced an Arthus-like reaction.

With animals sensitized to BSA, intestinal mucosal exposure to an unrelated antigen, egg albumin, did not induce increased neutrophil emigration into the intestinal lumen.

Although little apparent mucosal injury occurred 4 h after enteral exposure to BSA in immune animals, it seemed possible that a reaction analogous to a cutaneous Arthus may take longer to become manifest in the intestine. To determine whether or not this was so, a 110-cm segment of jejunum, in pigs sensitized to BSA, was temporarily ligated with Doyan forceps and inoculated with BSA. Each 10-cm length of intestine was exposed to 525 mg of BSA as in the previous experiments. After 4 h, large numbers of neutrophils emigrated into the intestinal lumen as described above. At this time, the Doyan forceps were removed and the gaseous anesthetic was discontinued. The animals recovered quickly, ate, drank, and appeared normal throughout the experimental period. Twenty-four hours after surgery, the BSA-exposed segment of small intestine appeared normal with no histological evidence of neutrophil accumulation or any other sign of inflammation.

To determine whether the antigen-induced neutrophil emigration might be mediated by antibody, pigs were passively immunized intravenously with anti-BSA serum, and the numbers of neutrophils emigrating into the intestinal lumen were evaluated.

Four hours after enteral exposure to BSA, the immune serum recipients had a three- to five-fold increase in the numbers of neutrophils in the intestinal lumen in comparison with the nonimmune serum recipients (Table 2). This response was approximately one-fifth of that observed in the actively immunized animals. In the recipients of nonimmune serum, no significant differences in the numbers of neutrophils resulted from any of the eight inocula. The intestinal and cutaneous histological changes, although quantitatively less pronounced, were qualita-
tively similar to those described for the actively immunized pigs.

In summary, these experimental findings suggest that emigration of neutrophils into the intestinal lumen can be an antibody-mediated, specific immune response that occurs in the absence of any persistent intestinal mucosal injury.

**DISCUSSION**

The intestinal tract encounters an intense, diverse, and persistent direct exposure to exogenous material. Intestinal mucosa is repeatedly exposed to dietary and normal microfloral antigens, and it has been shown that individuals will develop circulating antibodies to dietary antigens with no evidence of untoward effects after ingestion of the corresponding antigen (15). In view of the results reported herein, it seems possible that enteral antigens of all types may be involved in the regulation of neutrophil production by determining, in part, neutrophil loss from the body through the mechanism of immune-mediated enteroluminal neutrophil emigration. The extent of neutrophil loss would depend on the particular antigens present within the intestinal lumen and the immune state of the animal in relation to these antigens.

It is attractive to postulate that immune-mediated emigration of neutrophils into the intestinal lumen may be part of the acquired immune response by the intestine. There are at least two ways that such a process might have a protective function. First, if specific antibodies in intestinal secretions have opsonic capabilities, then the integrated action of specifically attracted neutrophils acting as phagocytes and specific antibodies acting as opsonins could be a factor in the elimination or control of specific microorganisms or harmful macromolecules in the intestinal lumen. Second, if large numbers of neutrophils, attracted by a specific antigen, simply disintegrate within the intestinal lumen, releasing their intracellular enzymes (1) and antibacterial substances (5, 8, 20), the offending antigen may be inactivated or destroyed. This would require that the released substances be active under the chemical and physical conditions of the intestinal lumen.

These suggestions might appear inconsistent with the observation by several workers (2, 4, 17) that passive enteral immunization protects against enteric bacterial infections. Under these conditions, we did not observe an increased neutrophil emigration. However, both observations are consistent with the concept that the acquired enteric immune response depends on the fate of enteral antigen. The primary exposure of the intestinal mucosa to an antigen can elicit both a local and a systemic immune response (11, 13, 14). The response to the secondary exposure of the intestinal mucosa to the same antigen may then depend on its fate in the intestine. If enough specific secretory IgA is present, the antigen may remain in the intestinal lumen. In the case of bacteria, these could be effectively "neutralized" by preventing colonization of the mucosal surface (19), and viruses and toxins could be neutralized (7, 12). If, however, the antigen passes beyond the epithelial surface, either because of inadequate levels of local antibody or because it is invasive, then a systemic immune response, like the neutrophil response described in this paper, could result and eliminate the offending antigen.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**

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