Difference between Bacterial and Food Antigens in Mucosal Immunogenicity

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The mucosa-associated lymphoid tissue may deviate from its systemic counterpart in being able to discriminate between microbial and nonmicrobial antigens. To study this, the systemic and mucosal antibody responses to bacterial and food antigens were followed in parallel in female rats during two pregnancies and lactation periods. Germfree rats were monocolonized with an Escherichia coli O6K13H1 strain, and their diet was switched to pellets containing large amounts of ovalbumin and β-lactoglobulin. Antibodies against O6 lipopolysaccharide already appeared in serum and bile 1 week after colonization, and those against type 1 fimbriae appeared a few weeks later. Serum immunoglobulin G antibodies against the E. coli enzyme β-galactosidase were found in moderate titers in all rats after 16 weeks of exposure. In contrast, few rats had detectable antibody levels against the dietary proteins ovalbumin and β-lactoglobulin in serum or bile even after 16 weeks of exposure. In the milk, antibodies against E. coli β-galactosidase and type 1 fimbriae reached the highest titers, while moderate titers were found against the food antigens and against O6 lipopolysaccharide. The difference in immune reactivity against bacterial versus dietary antigens was not likely due to insufficient amounts of the latter reaching lymphoid tissue, since (i) uptake studies indicated that ovalbumin was more efficiently taken up than endotoxin and (ii) the same difference in antigenicity between ovalbumin and E. coli was seen after immunization directly into Peyer’s patches. We therefore suggest that a prerequisite for a strong mucosal antibody response is that the antigen be encountered by the gut-associated lymphoid tissue within microorganisms capable of stimulating antigen presentation.

The intestine is the most important port of entry for foreign antigens and also contains the majority of the lymphoid effector cells of the body, collectively referred to as the gut-associated lymphoid tissue (GALT) (1). The antigens delivered via the intestine range from harmless food constituents via gut commensal microbes to enteropathogenic microorganisms.

The immunogenicity of intestinal bacteria depends on the degree of contact with lymphoid tissue (16, 29). Thus, killed bacteria are inefficient antigens when given perorally, because they are rapidly dislodged from the mucosa (30), but strongly antigenic when injected directly into intestinal lymphoid tissue (6). The antibody response to bacteria efficiently colonizing the gut mucosa consists of both a rapid secretory antibody response, dominated by immunoglobulin A (IgA), and a strong serum antibody response (13).

In contrast, food proteins such as ovalbumin, β-lactoglobulin, and casein mainly seem to stimulate serum IgG antibodies (31), especially those of the IgG2 and IgG4 subclasses (19). In some animal species, peroral administration of soluble protein instead gives rise to immunological tolerance (33). Theoretically, however, the GALT should encounter doses sufficient to be antigenic, since a measurable fraction of food proteins escapes proteolytic breakdown in the intestinal lumen and can be detected in the circulation antigenically intact (18, 34, 35).

To study a possible dissociation in mucosal responsiveness to dietary versus bacterial antigens, germfree female rats concomitantly were monocolonized with an Escherichia coli strain and had their diet exchanged for pellets containing large amounts of ovalbumin and β-lactoglobulin. The antibody responses to three bacterial antigens (O6 lipopolysaccharide [LPS], type 1 fimbrial antigen, and β-galactosidase) and two dietary antigens (ovalbumin and β-lactoglobulin) were monitored in serum and bile and in milk obtained from two consecutive lactation periods.

MATERIALS AND METHODS

Germfree, monocolonized, and conventional rats. Rats of the AGUS strain (12) were kept under germfree conditions as described earlier (14, 15). The AGUS strain of albino rats is of Long Evans origin and has been bred under germfree conditions for over 30 years. In the isolators, the rats were kept in metabolic cages on raised screens until late pregnancy, when they were transferred to plastic cages with wood shavings as embedding material.

Germfree rats were monocolonized with E. coli strain O6K13H1 (Culture Collection of University of Göteborg, no. 20561). This strain was originally isolated from a patient with cystitis and carries mannose-specific type 1 fimbriae, as evidenced by mannose-sensitive agglutination of guinea pig erythrocytes (10). A frozen stock of the strain was thawed, inoculated onto nutrient broth, and cultured overnight. Approximately 0.1 ml of broth culture was injected with a plastic syringe into the mouth of each rat, and drops of the broth were dispersed onto the fur of the animals and on the food pellets.

The rats were conventionalized by transferring the cages from the isolators into a conventional animal house.

Food pellets. Before the start of the experiment, the rats were fed commercially available pellets, R3 (Ewos, Södertälje, Sweden). This food, which lacks egg as well as milk

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constituents, was used as a basis for the fabrication of pellets containing ovalbumin and β-lactoglobulin. The protein sources of R3 were decreased to 50% and replaced by 25% egg powder and 25% milk whey powder (Lactolac 70; Borregard Trd, Göteborg, Sweden). The vitamin concentration was equal to that of the original pellets, except for the level of biotin, which was increased 10 times to compensate for binding activity in egg protein (G. Westerholm, personal communication). Cellulose starch was included to give the same fiber/protein ratio as in R3. The concentrations of ovalbumin and β-lactoglobulin in the egg white and milk whey powder, respectively, were determined by rocket immunoelectrophoresis (36), with purified ovalbumin and β-lactoglobulin (Sigma Chemical Co., St. Louis, Mo.) as standards, in a 1% (wt/vol) agarose gel containing 1% (vol/vol) rabbit anti-ovalbumin (Dakopatt AS, Copenhagen, Denmark) or rabbit anti-β-lactoglobulin (Nordic, Tilburg, The Netherlands). By this method, the concentration of ovalbumin in the egg powder was estimated to be 65% and the concentration of β-lactoglobulin in the milk whey powder estimated to be 60%. With a calculated food consumption of 10 to 15 g of food per day (Westerholm, personal communication), the daily intake of ovalbumin and β-lactoglobulin would be approximately 0.8 g of each per day but somewhat more for pregnant and lactating dams.

Experimental design and collection of samples. The experimental design is outlined in Fig. 1. One group of rats (10 females and 6 males) was monocolonized on day 0 with E. coli O6K13H1, while the other group was left germfree (5 female rats plus 3 male rats). On the same day, the rats in both groups were mated. The rats in both groups were given pellets containing ovalbumin and β-lactoglobulin (OB pellets) since day -2. Male rats were taken out from the isolators for collection of bile and serum samples after 1, 2, and 3 weeks. Five of the female rats in the colonized group and all five female rats in the germfree group were sacrificed after 5 weeks, after bile, serum, and milk had been obtained. The remaining five female rats were conventionalized and mated a second time for collection of bile, serum, and milk from a second lactation period.

For collection of samples the animals were anesthetized with ether or mebunal (60 mg/kg). Bile was drained from the common bile duct, and milk was obtained by mild water suction after the intraperitoneal injection of 5 IU of Syntocinon (Sandoz, Basel, Switzerland). Serum was collected by heart puncture. From the rats sacrificed during the second lactation period, the spleen and the mesenteric lymph nodes were assayed for antibody-forming cells by the ELISPOT method (see below).

Bacterial characterization. When removed from the isolators, the rats were tested for bacterial contamination of the fur, mouth, and rectum. Samples from the bacterial flora of the gastrointestinal tract were obtained from the stomach, duodenum, jejunum, ileum, cecum, and colon, using a cotton-tipped culture swab that was pressed against the gut contents. Colonization of the Peyer's patches was assessed by making an incision in the serosal wall of a patch and pressing a sterile swab against the exposed interior. The swabs were put in sealed tubes with modified Stuart transport medium and kept at room temperature in these tubes until analysis. The swabs were streaked on Drigalski agar plates and, for the conventionalized animals, also on blood agar, and bacterial growth was recorded after aerobic culture overnight at 37°C. Bacteria from the conventionalized animals were characterized by colony morphology, Gram staining, and biotype, and the gram-negative bacteria were O- and K-antigen typed.

Cultures of E. coli were assayed for the presence of mannose-specific type 1 fimbriae in the following manner. Three to five colonies were picked with an inoculating loop, suspended in phosphate-buffered saline (PBS), and mixed on microscope slides with human or guinea pig erythrocytes in either 0.15 M PBS, pH 7.4, or 2.5% (wt/vol) methyl-α-D-mannoside in PBS. The slides were gently tilted for 3 min,
Measurement of antibody titers. Antibodies were measured by enzyme-linked immunosorbent assay (ELISA) (11). Polyvinyl micro-dilution plates (MIC-2000; Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 10 μg of ovalbumin, 5 μg of β-lactoglobulin, 5 μg of E. coli β-galactosidase, and 2 μg of O6 LPS prepared by phenol-water extraction (39) or 5 μg of type 1 fimbrial antigen per ml, all diluted in PBS. The type 1 fimbrial antigen was kindly provided by C. Brinton, Pittsburgh, Pa. The serum, milk, or bile samples were diluted from 10⁻¹ to 10⁻⁵ in PBS with 0.05% Tween 20 (Kebo, Goteborg, Sweden) and allowed to bind the immobilized antigen for 4 h at room temperature. Goat anti-rat IgG (Organon Teknika, Malvern, Pa.), goat anti-rat IgA (Nordic), and affinity-purified goat anti-rat IgM (Organon Teknika), all Fc specific, were conjugated to alkaline phosphatase (Sigma) with glutaraldehyde. Anti-immunoglobulin conjugates were added to the washed plates and allowed to bind overnight at room temperature. After washing, bound enzyme activity was detected by addition of the substrate p-nitrophenyl phosphate (1 g/liter) in diethanolamine buffer (1 M; pH 9.8). The A₄₀₅ was recorded after 100 min with a Titrertec Multiscan photometer (Flow Laboratories, Inc., McLean, Va.), and the titer of any sample was defined as the reciprocal of the dilution giving an absorbance of 0.100 over the PBS background. Against most antigens low activities of serum IgM antibodies (0.2 to 0.4 unit of absorbance in the 10⁻¹ dilution) were present in unimmunized rats. Therefore, for calculations of serum IgM antibody titers, the background was instead defined by a pool of serum from germfree rats (for antibodies against LPS and type 1 fimbrial antigen) or normal rat serum (for antibodies against ovalbumin and β-lactoglobulin).

ELISPOT assay for detection of antibody-secreting cells. The ELISPOT assay described by Czerkinsky et al. (4) was used to detect cells secreting antibodies against ovalbumin, β-lactoglobulin, and heat-extracted E. coli antigens after 16 weeks of bacterial colonization. Cell suspensions were prepared from mesenteric lymph nodes and spleen by perfusion of the organs with Hanks balanced salt solution while the organ was gently pressed with a pair of forceps. The cell concentration was adjusted to 10⁶/ml in Iscove medium supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 μg of gentamicin per ml. The cells were kept in this medium at room temperature for approximately 8 h during transportation before they were analyzed by the ELISPOT technique. Polystyrene 50-mm-diameter tissue culture dishes (Falcon 1006; Becton Dickinson Labware, Oxnard, Calif.) were coated with 100 μg of ovalbumin, β-lactoglobulin, or ferritin (all from Sigma) per ml or with heat-extracted antigen from E. coli O6K13H1 corresponding to 10⁷ bacterial per ml. Heat-extracted antigen was obtained from the supernatant of E. coli O6K13H1 bacteria boiled in PBS for 2 h and consists mainly of bacterial O antigen (17). The plates were washed three times with PBS and blocked with 10% fetal calf serum in PBS for 2 h to prevent nonspecific adhesion. Cell suspensions (undiluted or diluted 1:5 or 1:25) were added at a volume of 600 μl to the antigen-coated plates. After 4 h at 37°C, the plates were washed three times with PBS with 0.05% Tween 20, and peroxidase-conjugated rabbit anti-rat immunoglobulin (Dakopatt) was added, followed by a peroxidase-conjugated swine anti-rabbit immunoglobulin (Dakopatt). Two layers of peroxidase-labeled antibodies were used to enhance the darkness of the spots (5). The reaction was developed with 1.4-nitrophenylenediamine (0.5 gliter) and 0.01% (vol/vol) hydrogen peroxide in a molten 48°C 1% agar solution poured in a thin layer over the plates. The spots were read in a stereomicroscope equipped with vertical white light under ×6 magnification (Zeiss).

Quantification of uptake of LPS versus ovalbumin. The uptake of bacterial antigen (endotoxin) compared with ovalbumin was studied in six rats. These rats were reared germfree and were taken out of the isolator just before the experiment and placed in cages with sterilized wood shavings. Three of the rats were given 100 mg of ovalbumin and 5 × 10¹² Formalin-killed E. coli O6K13 in 2 ml of PBS; three rats were given 100 mg of ovalbumin and concentrated heat-extracted antigen from the same bacterial strain in the same volume. Serum was obtained by heart puncture after 1, 2, 4, and 6 h and assayed for concentrations of ovalbumin and endotoxin.

Ovalbumin was quantified in serum by using a sandwich ELISA. Microdilution plates (MIC-2000; Dynatech) were coated with rabbit anti-ovalbumin (Dakopatt) purified on ovalbumin coupled to CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden). Serum, diluted in twofold steps in PBS, was incubated in duplicate in the coated plates for 4 h, together with an ovalbumin standard. After washing, an alkaline phosphatase conjugate of the rabbit anti-ovalbumin was added. The reaction was developed with p-nitrophosphorylphosphate as described for the antibody ELISA. The limit of detection was 3 ng/ml.

Endotoxin was quantified by a quantitative chromogenic Limulus amoebocyte lysate assay (Coatest Endotoxin; Kabi-Vitrum, Stockholm, Sweden). The limit of detection under the conditions used was 10 pg/ml.

Immunization in Peyer’s patches and subcutaneous immunization. Conventional Sprague-Dawley rats of both sexes were immunized in the Peyer’s patches with 10 mg of ovalbumin or 10⁹ Formalin-killed E. coli O6K13H1 in PBS. The animals were anesthetized with ether, and their abdomens were opened with a midline incision. The Peyer’s patches were localized on the serosal side of the intestine, and the antigen was carefully injected into at least 10 patches along the intestine. At 8 to 10 days after the immunization, bile was drained from the common bile duct and assayed for antibodies by ELISA.

Conventional Sprague-Dawley or AGUS rats were immunized subcutaneously with antigens emulsified in Freund incomplete adjuvant. One group (Sprague-Dawley) received 1 mg of ovalbumin plus 1 mg of β-lactoglobulin; the second group (AGUS) received 1 mg of ovalbumin plus 1 mg of β-lactoglobulin and 10⁹ Formalin-killed O6K13H1 bacteria. The animals were bled 7 and 21 days after immunization, and sera were assayed for antibody titers against ovalbumin, β-lactoglobulin, and O6 LPS by ELISA.

RESULTS

Bacterial colonization of rats. E. coli O6K13H1 rapidly colonized the entire gastrointestinal tracts of the rats and persisted throughout the period of monocolonization. E. coli could also be recovered from Peyer’s patches in some of the monocolonized rats. The conventionalized rats all still carried E. coli O6K13 in the distal parts of the intestine (cecum and colon and sometimes the lower ileum), but not in the duodenum or jejunum. No other aerobic or facultatively anaerobic gram-negative bacteria appeared to have colo-
FIG. 2. Antibody titers in serum and bile against LPS (●), type 1 fimbrial antigen (■), ovalbumin (○), and β-lactoglobulin (□) as measured by ELISA. The symbol denotes the median and the vertical bar indicates the range. For antibodies against bacterial antigens, only colonized or conventional animals are included; for antibodies against food antigens, germfree animals are also included.
nized the gastrointestinal tract upon conventionalization, since monomorphic cultures, which all consisted of a strain typed as O6 K13, were obtained on Drigalski agar plates cultured under aerobic conditions. On blood agar, Staphylococcus simulans and Enterococcus faecium were recovered from ileum, cecum, colon, and sometimes jejunum of the conventionalized rats. Bacterial cultures from rats kept under germfree conditions were uniformly negative.

All colonies of E. coli O6K13 expressed type 1 fimbriae as evidenced by a mannose-sensitive agglutination of guinea pig erythrocytes after one subculture on Drigalski agar.

**Antibody responses to bacterial and food antigens in serum and bile.** The antibody responses to two bacterial and two food antigens (O6 LPS, type 1 fimbriae, ovalbumin, and β-galactosidase) in serum and bile are shown in Fig. 2. The response against bacterial LPS was extremely rapid, as reflected by the appearance of antibodies of IgG, IgM, and IgA isotypes in bile and of IgG and IgM isotypes in serum by 1 week after colonization. A transient IgM response against LPS was seen in bile during the first week after colonization; it disappeared upon continuous exposure. The response against type 1 fimbriae appeared 1 to 3 weeks later and the titers were lower than those against O6 LPS. A slowly increasing IgA response in serum was detected against both LPS and type 1 fimbriae. Rats kept under germfree conditions did not have any detectable antibodies against LPS of type 1 fimbrial antigen.

The antibody activity in serum and bile was also assayed against the intracellular bacterial enzyme β-galactosidase (data not shown). No antibody response was detected during the first 5 weeks. After 16 weeks, however, all colonized rats had serum IgG antibodies (median, 1.6; range, 1.2 to 1.8), but no IgM antibodies. Of two bile samples assayed from the rats colonized for 16 weeks, one had IgA antibodies of low (1.1) titer and the other had IgG antibodies of low (1.0) titer.

In contrast to the bacterial antigens, ovalbumin and β-lactoglobulin induced only very weak antibody responses in serum and bile. Low titers of IgM antibodies against ovalbumin could be detected in the sera of five of eight rats and in the bile of two of eight rats after 16 weeks of exposure. One rat had detectable IgA antibodies against ovalbumin in bile at this time. All bile and serum samples were negative for antibodies against β-lactoglobulin.

**Antibodies against food and bacterial antigens in milk.** The antibody response in milk differed rather markedly from that in serum and bile, but the strongest antibody response was also seen against bacterial antigens (Table 1). The antibodies against O6 LPS were of rather low titers and had disappeared at the time of the second lactation period. Instead, antibodies against E. coli β-galactosidase appeared; in some animals, these were of very high titers. The antibody response against type 1 fimbriae was intermediate between that against O6 LPS and β-galactosidase.

Antibodies against the food antigens were somewhat more frequently found in milk than in serum or bile. These antibodies did not appear until the second lactation period, but then four of four rats responded with IgM anti-ovalbumin and three of four responded with IgA anti-ovalbumin antibodies. One rat had IgA anti-β-lactoglobulin antibodies in milk. Milk IgG antibodies against food proteins were, however, lacking in all animals.

**TABLE 1. ELISA antibody titers against food and bacterial antigens in milk from the first and second lactation periods of rats colonized by E. coli O6K13H1 and fed ovalbumin- and β-lactoglobulin-containing pellets**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Median log ELISA antibody titer (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st lactation period</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>Food</td>
<td></td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>&lt;1</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>&lt;1</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
</tr>
<tr>
<td>O6 LPS</td>
<td>1.6 (&lt;1-3.8)</td>
</tr>
<tr>
<td>Type 1 fimbriae</td>
<td>1.8 (1.7-2.0)</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* Values represent the log_{10} antibody titers as measured by ELISA after 5 and 16 weeks of intestinal colonization with E. coli and exposure to approximately 1 g of dietary ovalbumin and β-lactoglobulin per day. The median and range represent five rats from the first and four rats from the second lactation period. All milk samples were collected on day 12 after parturition.

![FIG. 3. Antibody-producing cells in spleen (Sp) and mesenteric lymph nodes (MLN) against heat-extracted antigen from E. coli O6K13H1 (coli), ovalbumin (ova), β-lactoglobulin (β-1), and horse spleen ferritin (fer) as quantified by ELISPOT. Symbols: ○, germfree animals; ●, conventionalized animals. Each symbol denotes one rat, and the horizontal bar denotes the median.](http://iai.asm.org/)
MUCOSAL IMMUNOGENICITY OF BACTERIAL AND FOOD ANTIGENS

TABLE 2. ELISA antibody titers in bile after immunization with ovalbumin or whole bacteria into Peyer’s patches

<table>
<thead>
<tr>
<th>Immunization antigen</th>
<th>Detection antigen</th>
<th>No. of rats</th>
<th>Median log ELISA antibody titer (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin (10 mg)</td>
<td>Ovalbumin</td>
<td>17</td>
<td>&lt;1 (&lt;1-2.0) 1 (&lt;1-1.7) 1 (&lt;1-2.8)</td>
</tr>
<tr>
<td>Whole E. coli 06K13H1 (10^8)</td>
<td>O6 LPS (heat-extracted antigen)</td>
<td>8</td>
<td>2.9 (2.5-3.7) 3.7 (3.6-4.4) 3.5 (2.2-4.0)</td>
</tr>
</tbody>
</table>

*Median and range of log ELISA antibody titers against different antigens 9 days after immunization with 10 mg of ovalbumin or 10^8 Formalin-killed E. coli in PBS into Peyer’s patches. The log ELISA titer was defined as the negative value of the log dilution giving an absorbance of 0.1 above bile from nonimmunized controls. Data from immunization with whole bacteria are from reference 6.*

*The fractions of rats with antibody titers above background were as follows: IgG, 6/17; IgM, 4/17; IgA, 4/17 (ovalbumin group).*

Antibody-forming cells against food antigens and bacterial antigens. Sixteen weeks after colonization, the presence of antibody-forming cells against food antigens and E. coli O antigen in spleens and mesenteric lymph nodes was tested by the ELISPOT method. All rats had considerable numbers of antibody-producing cells against E. coli in the spleen, but lower numbers in the mesenteric lymph nodes (Fig. 3). No spots were detected against E. coli antigens in the sterile rats. The cells reactive against food antigens were fewer in number, sometimes not above the numbers for the unrelated antigen ferritin, and were more often found in mesenteric lymph nodes than in the spleen.

Uptake of ovalbumin and endotoxin given intragastrically to sterile rats. The uptake into the circulation of ovalbumin and endotoxin was studied in rats given a mixture of either whole bacteria and 100 mg of ovalbumin or heat-extracted antigen and 100 mg of ovalbumin. The endotoxin dose given to each animal was approximately 4 mg for those receiving whole bacteria and 1.5 mg for those receiving heat-extracted antigen, as assayed by the Limulus test.

Ovalbumin was detected in the circulation of all six rats, with peak concentrations 1 h after feeding (median, 23 ng/ml; range, 8 to 50 ng/ml). After 2 h, the median concentration was 8 ng/ml, and after 4 h, it was 12 ng/ml. At 6 h, three rats had levels below detection (3 ng/ml); the other three also had decreased levels compared with levels at 4 h.

Of the 24 samples, 3 showed endotoxin activity over the detection limit of 10 pg/ml but below 20 pg/ml. Two of these were from rats given whole E. coli (one rat, 1 h after feeding; in the other, 6 h after feeding). The third was the 4-h sample from a rat given heat-extracted E. coli antigen.

Based on the peak median concentration of ovalbumin in serum, 23 ng/ml, and a serum volume of 10 ml, the relative fraction in serum relative to the gastrointestinal tract at this time would be 23 × 10^-9 × 10/100 × 10^-3 = 2 × 10^-6. By analogy, assuming a peak median concentration of endotoxin in serum of 10 pg/ml, the fraction of endotoxin in serum would be 10 × 10^-12 × 10/4 × 10^-3 = 2 × 10^-8 for animals given whole bacteria and 100 times 10^-12 × 10/1.5 × 10^-3 = 6 × 10^-8 for those given heat-extracted antigen. Thus, under the above conditions, ovalbumin was taken up 10 to 100 times more efficiently than LPS.

Antibody response in serum and bile after immunization into Peyer’s patches. Rats immunized directly into the Peyer’s patches with 10 mg of ovalbumin dissolved in PBS showed a very weak antibody response in serum and bile (Table 2). In contrast, immunization in the Peyer’s patches with Formalin-killed E. coli was an effective means of stimulating antibodies in bile.

Antibody response in serum after subcutaneous immunization. When protein antigens and whole bacteria were given as a subcutaneous injection, however, the protein antigens were quite efficient (Table 3). There was no difference in the response to ovalbumin or β-lactoglobulin when whole Formalin-killed bacteria were included in the immunization mixture. The response to O6 LPS was moderate, even though the IgM response to this antigen was relatively better than that to ovalbumin and β-lactoglobulin.

DISCUSSION

In this study, a pronounced difference in immunogenicity was found between dietary proteins and antigens in bacteria colonizing the intestine, antibodies being detected at higher titers against all bacterial antigens studied than against the food antigens. In the cases of O6 LPS and type 1 fimbrial antigen, the response was also much more rapid than that to the food antigens, especially that to LPS, which was detected in both serum and bile within 1 week after colonization.

The difference in immune response against bacteria versus food proteins was not likely due to the latter being absorbed intact in quantities too small to stimulate the GALT, for three reasons. First, the animals were fed about 1 g each of the pure antigens ovalbumin and β-lactoglobulin. This equals...

TABLE 3. ELISA antibody titers in serum 7 and 21 days after subcutaneous injection with different antigens

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>Immunizing antigen (1 mg)</th>
<th>Median log ELISA antibody titer against:</th>
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<tbody>
<tr>
<td></td>
<td>Ovalbumin</td>
<td>β-Lactoglobulin</td>
</tr>
<tr>
<td></td>
<td>7 days</td>
<td>21 days</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
<td>10</td>
<td>Ovalbumin, β-lactoglobulin</td>
<td>1.7</td>
</tr>
<tr>
<td>12</td>
<td>Ovalbumin, β-lactoglobulin, E. coli (10^8)</td>
<td>2.1</td>
</tr>
</tbody>
</table>

*Median log ELISA antibody titers of the IgG and IgM isotypes in serum 7 and 21 days after subcutaneous immunization with antigens emulsified in Freund incomplete adjuvant (oil emulsion). The first group were of the Sprague-Dawley strain and received a mixture of ovalbumin and β-lactoglobulin. The second group were of the AGUS strain and received a mixture of ovalbumin, β-lactoglobulin, and Formalin-killed whole E. coli 06K13H1. ND, Not determined.*
roughly the total dry weight of bacteria in the gastrointestinal tract of the rat and thus surpasses by a factor of 10 to 1,000 the amounts of individual bacterial antigen present. Second, uptake studies indicated a better uptake of antigenically intact ovalbumin than Limulus-reactive LPS over the intestinal epithelium. For intact macromolecules such as proteins (of food or bacterial origin), the fraction taken up antigenically intact has been calculated to be around $10^{-3}$ to $10^{-4}$ of the amount given intragastrically (22, 35). There is no indication that bacterial macromolecules per se are more efficiently taken up than, e.g., dietary proteins. Translocation, i.e., the uptake of intact particles such as whole bacteria or latex particles over the intestinal epithelium (37), is a much rarer event than uptake of intact macromolecules, the fraction of intestinal bacterial translocated being in the range $10^{-6}$ to $10^{-7}$ (2, 38). Third, the same striking difference in antigenicity between ovalbumin and E. coli bacteria was seen after injection directly into the Peyer's patches, an immunization regimen which bypasses all possible differences in uptake over the epithelium.

The strong antigenicity of whole E. coli compared with soluble proteins was not seen when these antigens were administered subcutaneously, confirming earlier findings of a rather moderate antigenicity of LPS delivered parenterally (8). We therefore suggest that the GALT differs from its peripheral counterpart in that it discriminates between bacterial antigens and other soluble proteins. In accordance with our findings, live respiratory syncytial virus given intratracheally or perorally to rabbits stimulates secretory IgA, whereas human serum albumin administered to the same sites only stimulates serum IgG (27). Similarly, an increased antigenicity of cholera toxin, especially crude preparations contaminated with, e.g., LPS as compared with purified toxoid, is only seen after peroral exposure, not after systemic immunization (28).

If the GALT discriminates between dietary proteins and bacterial antigens, two possibilities exist. (i) Bacterial antigens may in themselves possess certain physicochemical characteristics which make them strongly stimulatory to B cells, T cells, or antigen-presenting cells in the GALT. (ii) Bacterial antigens do not all possess special antigenic characteristics, but antigenicity is conferred on them by the "bacterial context" in which they are presented to the GALT. The second hypothesis is favored by the high antibody titers found against the intracellular bacterial enzyme β-galactosidase, an intracellular protein of moderate molecular weight. A parallel is the finding of high levels of antibodies against bacterial IgA1 proteases in human milk and serum (20), although the actual concentrations of these enzymes on the mucosa probably are very low.

If the bacterial context confers antigenicity on any substance within the bacterial cell, the discrimination must be performed by a cell subset which encounters intact bacteria, e.g., phagocytes which also present the antigens to the immune effector cells. In fact, most antigen-presenting cells in the intestinal lamina propria are phagocytic (23) and are related to macrophages (25), whereas in systemic lymphoid tissue dendritic cells without the capacity to phagocytose particles are the most efficient antigen presenters (32). A mechanism whereby microorganisms, but not, e.g., soluble proteins, could activate antigen presentation could be their stimulation of interleukin production. The production of both interleukins 1 and 6 by macrophages is induced by LPS (3, 9), and these may be necessary for stimulation of an immune response (26). In contrast, presentation of antigen by major histocompatibility complex class II-positive cells, without a concomitant stimulation of T cells by interleukins, may be tolerogenic (21, 24).

Although stimulation by antigen in the GALT leads to a disseminated mucosal immune response which is reflected in both bile and milk, the antibody pattern differs markedly between these two secretions. Thus, after injection of whole bacteria into the Peyer's patches, anti-LPS antibodies localize primarily in the bile and antifimbrial antibodies localize in the milk (7). Similarly, in this study, low titers of antibodies against E. coli O6 were found in milk compared with those in bile, while antibodies against the food proteins and against fimbriae were more equally distributed in milk and bile. These findings will be published in a separate report (U. I. H. Dahlgren et al., manuscript in preparation).

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