ORAL VACCINATION

Identification of Classes of Proteins that Provoke an Immune Response upon Oral Feeding

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The induction of serum or mucosal antibody responses to orally administered antigens is often difficult and generally requires the administration of relatively large quantities of antigen, as the amount of antigen that is actually absorbed and capable of eliciting an immune response is usually low. As a consequence, the amount of antigen required for oral immunization generally far exceeds that required for parenteral induction of systemic immunity. Furthermore, the oral presentation of the large quantities of antigen required to produce an immune response often leads to the simultaneous induction of systemic tolerance (1-7).

Evidence to date suggests that in general the mechanism by which antigen is taken up by the small intestine is primarily via nonspecific sampling of the contents of the gut lumen by M cells that overlie the Peyer’s Patches and other lymphoid clusters of the gut-associated lymphoid tissue (GALT) (8). The subsequent sensitization of local lymphocyte populations leads to the generation of local IgA immune responses plus the sensitization of IgG and IgE suppressor cells with concomitant suppression of serum IgG and IgE responses (1-7).

During some recent experiments (unpublished observations), we noticed that feeding mice small quantities of Escherichia coli pili elicited both a significant serum antibody response to the pili, as well as a concomitant response in the intestine. Feeding similar quantities of BSA did not elicit any such antigen-specific serum or intestinal response.

The question arose then as to whether there exist any other antigens apart from the E. coli pili that exhibit the ability to specifically prime the mucosal immune system upon oral administration and/or to stimulate the humoral immune response in a dose-dependent manner, without inducing systemic tolerance and without the need for excessive doses of antigen.

With this objective in mind we decided to investigate the potential of certain adhesive molecules, which have been implicated in the initial attachment of a number of intestinal pathogens, to stimulate the immune response when orally...
administered. The surface antigens that confer adhesive properties to a number of strains of enterotoxigenic E. coli (ETEC) have been identified as nonflagellar, filamentous proteinaceous appendages, called fimbrae or pili (9). Examples include the CFA I and CFA II pili of human ETEC strains and the K88, K99, F41 and 987P pili of animal ETEC strains (10–14). In addition, we have examined the ability of a number of other proteins that have no apparent role in colonization to prime the immune system upon oral feeding. These antigens included a number of lectins, a serotypic antigen of Salmonella typhimurium (the type I flagella), inactivated flu virus, and S. typhimurium endotoxin (LPS). Oral priming was compared to the response generated by wholly intramuscular challenge.

In the course of these studies we have been able to identify classes of antigens that when orally administered to an animal at low doses were effectively taken up by the cells of the gastrointestinal mucosa and were then able to elicit serum and/or secretory antibodies without the induction of systemic tolerance.

Materials and Methods

Materials. The various lectins and BSA were purchased from Sigma Chemical Co., St. Louis, MO. Inactivated influenza vaccine was purchased from the Commonwealth Serum Laboratories (Melbourne, Australia).

Bacterial Strains and Media. E. coli strains overproducing K99, 987P, or the E. coli labile toxin B subunit (LTB) were constructed at Biotechnology Australia Pty. Ltd., and were the generous gifts of Dr. Susan Clark. Cultures were grown at 37°C with shaking in Luria broth (LB). The strain expressing LTB was induced with 1 mM isopropyl-thio-n-D-galactopyranoside (IPTG). S. typhimurium was grown at 37°C with shaking in LB plus 0.2% glycerol.

Pili Preparation. E. coli cells expressing pili were harvested during the logarithmic phase of growth. Cultures were heated at 60°C for 30 min, after which the cells were pelleted by centrifugation (3,000 g, 30 min, 4°C). The supernatant was examined for pilic content by 12.5% SDS-PAGE using a modification of the method of Laemmli (15, 16).

K99 pili were purified by adjusting the culture supernatant to pH 9.7 with 10 N NaOH and stirring at room temperature (RT) for 10 min. The resultant precipitate containing pili was recovered by centrifugation (3,000 g, 30 min 4°C) and resuspended in 100 ml distilled water (dH2O) and the pH was lowered to 7.2 using 1 N HCl. This procedure was repeated twice.

987P pili were purified as detailed above except that pili precipitation was achieved by adjusting the pH to 3.9 with glacial acetic acid.

Hydroxyapatite Chromatography. Hydroxyapatite (HA) (DNA grade Bio-Gel HTP; Bio-Rad Laboratories, Richmond, CA) was gently swollen in an excess of dH2O and after a brief period (~2 min) fines were decanted gently. Fresh dH2O was added and used to gently resuspend the gel, after which fines were decanted again. This procedure was repeated several times. A column (30 x 5 cm) was filled with a slurry of ~70% HA and allowed to settle by gravity. Tight packing was then achieved by passing dH2O through the column at a flow rate of 16 ml/h until the gel bed surface was stationary. Samples (100 ml) of either K99 or 987P pili were applied at flow rates not exceeding 30 ml/h. The column was then washed with dH2O until no protein was detected in the flow through at 280 nm. Pili were eluted at a flow rate of 30 ml/h using a linear gradient of 15–250 mM sodium phosphate buffer, pH 7.5. Fractions were collected and examined by SDS-PAGE. The pilus peak was recovered and pooled.

Pooled fractions of the K99 and 987P pili after HA chromatography were reprecipitated with NaOH (by adjusting to pH 9.7) or glacial acetic acid (adjusting to pH 3.9) respectively. After centrifugation (3,000 g, 10 min), the pellets containing pili were resuspended in 50 mM citrate buffer, pH 5.5 (K99 pili) or 50 mM Tris-HCl, pH 8.5 (987P pili), before
loading on the ion-exchange columns equilibrated with the same buffers. K99 and 987P pili were loaded onto carboxymethyl (CM) and DEAE columns respectively at a flow rate of 100 ml/h, washed with 2 volumes of loading buffer and the pili were eluted using a linear gradient from 10 mM to 0.5 M NaCl in the equilibration buffers. Fractions were examined by SDS-PAGE for protein content and LPS contamination, according to the method of Tsai and Frasch (17).

**LTB Purification.** 3 liters of culture supernatant were diluted to 6 liters with dH2O. The pH was adjusted to 6.5 with glacial acetic acid and loaded onto a 5 x 30 cm column of fast-flow CM-Sepharose equilibrated with 10 mM phosphate buffer, pH 6.5, at a flow rate of 1.2 l/h. The column was then washed with 400 ml of 10 mM phosphate buffer, pH 6.5, and bound protein was eluted with a linear gradient of 10-500 mM NaCl in 10 mM phosphate, pH 6.5. Fractions were collected and analyzed by SDS-PAGE, and the LTB peak was pooled. Finally the LTB pool was loaded onto a column of o-aminophenyl-

**Flagella Isolation and Purification.** Late log-phase cultures of bacteria (expressing both i and 1,2 flagellae) were pelleted by centrifugation (3,000 g for 15 min at 4°C). The cells were resuspended in saline and heated at 60°C for 30 min, followed by centrifugation (3,000 g, 10 min, 4°C). The supernatant was precipitated by adding a solution of 100% TCA (wt/vol) to give a final concentration of 10% (wt/vol) and spun for 10 min at 1,500 g at 4°C. The pellet was resuspended in a small volume of 1 M Tris, pH 8.8, and sonicated until dissolved. Ethanol was added to a final concentration of 80% (vol/vol) and the flagellae were spun down at 2,000 g, 10 min at 4°C. The pellet was resuspended in acetone, sonicated into suspension, and precipitated by centrifugation (5,000 g). Finally, the pellet was brought into solution by boiling in 10% SDS and 50 mM EDTA in 10 mM Tris-HCl, pH 8.0, before Sephacryl S-200 chromatography.

After boiling for 15 min the flagella were clarified by centrifugation for 5 min in a Beckman Instruments, Inc., Fullerton, CA, benchtop microfuge to remove nonsolubilized material. The supernatant was applied to a 2.5 x 80 cm column of Sephacryl-S200 (Pharmacia Fine Chemicals) equilibrated with 20 mM Tris, pH 8.8, 0.1% SDS, and 10 mM EDTA, and eluted using the same buffer. Fractions were collected and analyzed by SDS-PAGE. Finally, the flagella peak was pooled and precipitated with 10% (final concentration) TCA followed by centrifugation, and ethanol and acetone washes as described above. The final pellet was resuspended in dH2O.

**LPS Purification.** Overnight cultures of *S. typhimurium* were extracted (30 min RT) with 0.5 M CaCl2 in 20% ethanol (v/v) containing 100 mM citrate, pH 3.0, and 5% Zwittergent 3.12 (wt/vol) (Calbiochem-Behring Corp., San Diego, CA). Bacteria were pelleted by centrifugation (3,000 g, 10 min at 4°C) and the pellet was resuspended in 50 mM EDTA, pH 8.0. The suspension was stirred vigorously for 30 min at RT. After removal of the bacteria by centrifugation, ethanol was added to the supernatant to a final concentration of 75%. Protein material was pelleted and the supernatant adjusted to 90% ethanol. The precipitate that formed was pelleted and washed with acetone, reprecipitated, and finally resuspended in dH2O. The preparation was assayed for sugar content using the Anthrone reagent (18) and checked for the presence of contaminating proteins using SDS-PAGE. Commercial *E. coli* LPS (Sigma Chemical Co.) was used as a standard in both assays. Gels were stained for LPS using a silver stain according to the method of Tsai and Frasch (17).

**Preparation of Polysaccharide (PS).** Lipid A was cleaved from the *S. typhimurium* LPS preparation by incubating the LPS with 1 M glacial acetic acid and heating at 100°C for 3 h. Lipid A was then removed by centrifugation at 3,000 g for 10 min at 4°C. The supernatant was then lyophilized.

**Description of Purified Antigens.** SDS-PAGE analysis of purified K99 and 987P pili preparations revealed the presence of a single band migrating at 17,500 and 20,000 mol wt, respectively, under reducing conditions (Fig. 1). This agrees with the published data of Isaacson and others (19–22). The ease of precipitation of these proteins at pH 9.7 and
FIGURE 1. SDS-PAGE analysis of purified antigens. Purified proteins were examined by SDS-PAGE using a 14% polyacrylamide gel. LTB eluted from CM-Sepharose (a, lane 1) was passed down a column of agthio-galactose-Sepharose (Sigma Chemical Co.) and eluted with 0.1 M galactose. The flow-through contained no LTB (lane 2), while the B was eluted by galactose as a single protein band (lane 3, 50 μg). Purified 987P and K99 pili and flagella are shown in b (lanes 4-6, respectively; 25 μg). Bio-Rad Laboratories low-molecular weight markers are shown, with molecular weights (× 10^3). 3.9 (for K99 and 987P, respectively) suggests that the pl of these two proteins is around these ranges (see references 19, 21). Silver staining of the preparations showed them to contain little (<1 μg/100 μg protein) or no contamination with LPS.

Purified LTB and S. typhimurium flagella were also found to be free of contaminating LPS and to travel as monomers of apparent molecular weights of 12,500 and 52,000, respectively, when examined by SDS-PAGE under reducing conditions (Fig. 1). The flagellae preparation was found to be contaminated by a second, slower migrating band, which was found to be the alternate 1,2 flagellar phase. The main band was the i phase (results obtained by Western blot analysis and not shown here).

Silver-stained SDS-PAGE gels of purified LPS revealed no detectable protein contamination. Complex sugar content, as assayed by Anthrone reaction, was found to be 2 mg/ml. Lipid A–free polysaccharide was also found to contain 2 mg/ml polysaccharide and its failure to move on SDS-PAGE (as revealed in silver-stained gels) showed it to be free of contaminating lipid.

**Determination of the 987P Pilus NH₂-Terminal Sequence.** NH₂-terminal micro-sequencing was performed for us by Biotechnology Research Enterprises S.A. Pty. Ltd., Adelaide, South Australia. A 100 nmol sample of 987P pili purified as described above was assayed. The NH₂-terminal sequence of 987P is compared with the published sequence of a number of other published sequences and reveals no homology between it and the other molecules (Fig. 2) (9).

**Dinitrophyenlalation of Antigens.** K99 pili, LTB, and lectins were dinitrophenylated according to the method of Little and Eisen (23). Briefly, carriers (in 0.1 M sodium carbonate/bicarb buffer, pH 9.5) were reacted with a 0.1 M solution of dinitrofluorobenzene (DNFB, Sigma Chemical Co.) in acetone overnight at RT. The proteins were then dialyzed extensively against the coupling buffer. Previous studies by us have shown that 987P pili have no free amino groups exposed for coupling so a diamino spacer was first linked to the free carboxyl moieties of the protein as follows: 10 mg of purified 987P pili were precipitated at pH 3.9 by the addition of glacial acetic acid. The pili were removed by centrifugation at 3,000 g, 10 min at 4°C. The pellet was resuspended in dH₂O and the pH raised to 6.5 with 1 N NaOH. The pilus solution was then reacted with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl (EDAC; Bio-Rad Laboratories, Richmond, CA), to a final concentration of 0.5 mM in the presence of 20 mM 1,2-diaminoethane, pH 6.5 (BDH Chemicals Ltd., Poole, England), overnight at room temperature (20–23°C). The amino-substituted 987P pili were dialyzed for 24 h against two changes of
MUCOSAL IMMUNOGENS

Type 1  Ala-Ala-Thr-Thr-Val-Asn-Gly-Gly-Thr-Val-His-Phe-Lys-Gly-
K88    Trp-Met-Thr-Gly-Asp-Phe-Asn-Gly-Ser-Val-Asp-Ile-Gly-Gly-
K99    Asn-Thr-Gly-The-Ile-Asn-Phe-Asn-Gly-Lys-Ile-Thr-Ser-Ala-
987P   Ala-Pro-Val-Glu-Asn-Asn-Thr-Cys-Gln-Ala-Asn-Leu-Asp-Phe-
Neisseria Phe-Thr-Leu-Ile-Glu-Leu-Met-Ile-Val-Ile-ile-Val-Gly-

0.1 M carbonate/bicarbonate buffer, pH 9.5, before being used in the subsequent conjugation steps. Lectin binding sites were protected during their reaction with DNFB by the addition of the lectin-specific sugars. Thus, 50 mM solutions of D-glucose, D-mannose, N-acetyl-D-galactosamine, D-galactose, N-acetyl-D-galactosamine, D-gal(1-3)-D-galNAc, and L-fucose, were added to lectins from the following sources: ConA, PWM, Lens culinaris, Helix pomatia, Glycine max, Arachis hypogea and Ulex europeus, respectively.

Antigen Administration. Female C57BL/6J mice (18–22 g) were obtained from the Animal Resources Center (Perth, Western Australia). All mice were starved for 3–4 h before oral or intramuscular administration of antigens. Mice were fed antigen at appropriate concentrations in 0.5 ml of 0.1 M carbonate/bicarbonate buffer, pH 9.5, using a specially prepared feeding needle. Parallel doses of antigen were injected intramuscularly, in 0.1 ml of sterile physiological saline, into the left hind leg. Groups of five mice receiving antigen either orally or intramuscularly were given two identical doses of antigen, on day 0 and day 14. A blood sample was taken (~0.5 ml) from the retro-orbital plexus on day 14 and day 21. Mice were then killed by cervical dislocation and gut washes performed on the small intestine in the following manner. The small intestine was carefully removed and 1 ml of washing buffer (50 mM Tris-HCl, pH 8.8, 0.9% NaCl, 50 mM EDTA plus 1.0% Tween 20) introduced into the lumen of the gut via a blunt-ended feeding needle. After gently kneading the intestine, the contents were squeezed out through forefinger and thumb. Gut washes so obtained were immediately centrifuged to remove debris and stored at −20 °C until assayed. Blood samples were allowed to clot at 4°C before removal of the serum and storage at −20°C.

Inhibition of the Immune Response by Cofeeding-specific Sugars. A number of sugars were tested for their ability to inhibit the immune response generated when the mucosal immunogens were administered orally. Immediately before feeding, LTB, 987P and K99 pili were mixed with 25 μmol of the various sugars. Mice were then fed, bled, and sera were assayed as described previously.

Enzyme-linked Immunosorbent Assay (ELISA). The ELISA for the determination of antibody titers was performed as described previously by Russell-Jones et al. (24). The assay was standardized so that 1 μg of specific antibody produced an ELISA titer of 10³ while 1 mg of specific antibody gave a titer of 10⁶.

Results

Identification of Molecules Active as Mucosal Immunogens. The potential of a number of molecules to stimulate the production of an immune response after oral administration was examined. The response generated by molecules with known binding activities was compared with the response seen after similar feeding of other molecules having no known mucosal binding functions.
TABLE I

Immune Reactions to Orally Presented Antigens

<table>
<thead>
<tr>
<th>Antigen used for immunization (20-μg dose)</th>
<th>Serum</th>
<th>Intestinal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgA</td>
</tr>
<tr>
<td>K99 pili</td>
<td>968 ± 120</td>
<td>&lt;4</td>
</tr>
<tr>
<td>987P pili</td>
<td>776 ± 64</td>
<td>10.8 ± 8.8</td>
</tr>
<tr>
<td>LTB</td>
<td>1,551 ± 211</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Flu vaccine</td>
<td>179 ± 54</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Flagella</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
<tr>
<td>LPS</td>
<td>12.1 ± 1.0</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Polysaccharide</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
<tr>
<td>BSA</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Con A†</td>
<td>666 ± 84</td>
<td>&lt;4</td>
</tr>
<tr>
<td>PW-mitogen†</td>
<td>641 ± 119</td>
<td>&lt;4</td>
</tr>
<tr>
<td>L. culinaris†</td>
<td>954 ± 48</td>
<td>&lt;4</td>
</tr>
<tr>
<td>H. pomatia†</td>
<td>591 ± 127</td>
<td>&lt;4</td>
</tr>
<tr>
<td>P. vulgaris†</td>
<td>1,378 ± 110</td>
<td>4.8 ± 2.5</td>
</tr>
<tr>
<td>G. max†</td>
<td>1,529 ± 65</td>
<td>3.1 ± 6.9</td>
</tr>
<tr>
<td>A. hypogea†</td>
<td>1,276 ± 242</td>
<td>&lt;4</td>
</tr>
<tr>
<td>U. europeus†</td>
<td>1,583 ± 94</td>
<td>&lt;4</td>
</tr>
</tbody>
</table>

* The reciprocal of the antiserum dilution that gave an ELISA reading of 0.5 after 45 min at 37°C. Each value represents the mean value obtained from 5 mice ± 1 SD. A titer of 1 in 1,000 corresponds to 1 μg of specific antibody per milliliter of serum.

† Each lectin was substituted with four DNP molecules/mole lectin. The antilectin response could not be measured directly, as sugar-specific binding to IgG interfered with the ELISA, and so the anti-DNP response is represented. Intestinal antibody titers were not measured for lectin-immunized animals (ND).

As can be seen in Table I, all of the proteins known to possess receptor binding activities were capable of eliciting serum antibody responses upon oral presentation. Thus K99 and 987P pili (bacterial adhesins), LTB (the GM-1-binding subunit of E. coli heat-labile toxin), the various lectins, as well as the inactivated flu vaccine (in which the hemagglutinin activity was still maintained), all elicited comparable serum antibody responses at the doses tested (Table I). This class of proteins will henceforth be referred to as mucosal immunogens. The marginally lower response elicited by the inactivated flu vaccine presumably reflects the smaller amount of hemagglutinin present in this preparation, as it is not a purified hemagglutinin vaccine but rather a whole virus preparation. Variable stimulation of intestinal antibodies occurred, but was only present upon feeding of the mucosal immunogens.

None of the other antigens tested were capable of eliciting a substantial serum response at the doses tested. Intramuscular injection of five of the proteins stimulated very similar antibody titers regardless of whether the protein was effective as a mucosal immunogen or not (Table II). Thus the serum titers to intramuscularly injected K99, 987P pili or LTB (all mucosal immunogens) varied little from that elicited by the intramuscular injection of flagellin or BSA (both shown to be ineffective orally).

Dose-response experiments comparing oral with intramuscular administration
TABLE II

Immune Reactions to Antigens Presented Intramuscularly

<table>
<thead>
<tr>
<th>Antigen used for immunization (20-μg dose)</th>
<th>Immune response* (day 21)</th>
<th>Serum</th>
<th>Intestinal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgG</td>
<td>IgA</td>
</tr>
<tr>
<td>K99 pili</td>
<td>1,024 ± 94</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
<tr>
<td>987P pili</td>
<td>1,552 ± 112</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
<tr>
<td>LTB</td>
<td>1,782 ± 100</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Flagella</td>
<td>1,595 ± 227</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
<tr>
<td>LPS</td>
<td>388 ± 58</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
</tbody>
</table>

* See Table I.

Figure 3. Effect of route and dose on the immune response to K99 pili (O, ), 987P pili (△, ▲) and LTB (□, ▣). Each antigen was injected intramuscularly (open symbols), or presented orally (filled symbols). Antibody titers are given as the serum IgG response (——); and the intestinal IgA response (— — —). Results are shown for the day 21 response and are expressed as the mean antibody titer of five mice.

Inhibition of the Immune Response to Oral Immunogens by Cofeeding Sugars. Stimulation of the immune response to orally presented LTB and K99 was completely abolished by cofeeding galactose or other molecules with close struc-
Inhibition of the Immune Response to Orally Administered Antigen by Co-feeding Certain Sugars

<table>
<thead>
<tr>
<th>Sugar (25-μM dose)</th>
<th>Immune Response to oral antigen (serum IgG)*</th>
<th>K99 pili</th>
<th>987P pili</th>
<th>LTB</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>968 ± 120</td>
<td>776 ± 64</td>
<td>1,351 ± 211</td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>1,782 ± 966</td>
<td>2,046 ± 894</td>
<td>6,208 ± 1,192</td>
<td></td>
</tr>
<tr>
<td>Galactose</td>
<td>65 ± 48</td>
<td>1,037 ± 526</td>
<td>4 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>84 ± 204</td>
<td>1,128 ± 662</td>
<td>5 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Mannose</td>
<td>1,176 ± 411</td>
<td>4,970 ± 2,270</td>
<td>4,056 ± 658</td>
<td></td>
</tr>
<tr>
<td>Melibiose</td>
<td>1,840 ± 208</td>
<td>1,243 ± 474</td>
<td>512 ± 76</td>
<td></td>
</tr>
<tr>
<td>Sorbitol</td>
<td>77 ± 179</td>
<td>1,389 ± 307</td>
<td>8 ± 1.2</td>
<td></td>
</tr>
</tbody>
</table>

A 20-μg dose of LTB or 987P or K99 pili was premixed with various sugars before feeding. Results represent the mean ELISA titers of serum of five mice. The serum response to these antigens is given.

* See Table I.

tural homology to galactose, such as lactose and sorbitol (Table III). Fructose, mannose, and melibiose did not inhibit the response to either LTB or K99 pili. The response to 987P pili could not be inhibited by any of the sugars tested.

Discussion

In contrast to parenteral immunization, attempts to orally immunize animals have generally involved the use of large quantities of antigen and have required as many as 8–10 repeated feedings of milligram quantities of antigen (1–7). The immunity induced by this regime has at best been poor, short lasting, and often confined to the mucosal immune system. Various explanations have been put forward for these anomalous findings, but the most widely held belief is that oral immunization of animals requires the antigen to be taken up by M cells of the Peyer's patches. Antigen is then processed by resident macrophages and presented to IgA helper cells and to IgG suppressor cells (6, 8, 25, 26). The result is the stimulation of a local IgA response and the suppression of a serum IgG response.

In this study we have shown that it is possible to effectively prime both the systemic and intestinal immune system by feeding of low doses of some antigens. In fact, many molecules that have the capacity to bind to the intestinal epithelium can also be transported across the epithelial barrier, to enter the circulation and elicit an immune response. Thus the oral feeding of two bacterial adhesins of enterotoxigenic E. coli, K99 and 987P pili, the binding subunit of E. coli heat-labile toxin (LTB), plus a number of lectins of varying binding specificities, were able to stimulate significant serum antibody titers after oral administration of the antigen (Table I and Fig. 3). In fact, this process of uptake appeared remarkably efficient as the response elicited to the orally presented mucosal immunogens was only slightly lower than the response generated by intramuscular injections of the same quantities of these antigens (Fig. 3). A slight variability in the ability of the mucosal immunogens to elicit an intestinal response was noted. For some reason, 987P pili stimulated a much higher intestinal response than any of the other antigens tested over doses tested.
Feedings of similar quantities of BSA, flagella, LPS, or polysaccharide (PS) induced no detectable antibody response either in serum or in intestinal fluid.

Binding of the mucosal immunogens to the intestinal mucosa appeared to be a specific phenomenon as the immune response to LTB and K99 could be completely abolished by cofeeding galactose, lactose, or sorbitol, but not by cofeeding any of the other sugars tested (Table III). The response to 987P pili was not affected by these sugars. The inhibition of an immune response to LTB and K99 pili seen when these molecules were cofed with galactose, lactose, and sorbitol is to be expected due to the similarities in structure of these compounds to galactose, which is claimed to be the specific sugar(588,679),(642,716) determinant on both the GM1 ganglioside to which LTB is known to bind (27–29), and on the GM2 ganglioside to which K99 is thought to bind (9). The receptor for 987P is yet to be characterized. Furthermore, the abolition of the response seen with these sugars suggests that binding to the mucosa is a specific, receptor-mediated reaction in which the mucosal immunogens bind to specific glycoproteins or glycolipids on the villous epithelium. In this respect, intestinal uptake of these antigens differs significantly from the antigen sampling mechanism suggested for the uptake of orally administered antigens (7, 8, 10, 11, 25).

It is interesting to compare these results with the work of Pierce and others (30–34) on the oral feeding of cholera toxin. Oral administration of relatively small quantities of this toxin, in the 10–100 μg range, results in the stimulation of relatively good antibody responses in both serum and secretions. Animals receiving larger doses of this toxin exhibit all the symptoms of a cholera infection (30). These results interalia, led to the popular notion that cholera toxin is a potent immunogen (31, 32). It must be noted however, that parenteral administration of this molecule does not elicit antibody responses that are significantly higher than that elicited by similar administration of other proteins such as BSA or keyhole limpet hemocyanin. When cholera toxoid, which is nontoxic, is orally administered it is much less efficient in provoking an antibody response. Thus, Pierce and Koster (33) required doses of 40 mg of toxoid to generate an immune response to the intraduodenal administration of this antigen. These experiments suggested that the toxicity of the intact toxin is required for its ability to prime the immune system orally. However, experiments using only the B subunit of cholera toxin (CTB) (34), suggest that this is not the case. Thus, when CTB was administered without the toxic A subunit it was still effective in priming the immune system upon oral challenge. It would seem, therefore, that the enhanced ability of cholera toxin to prime the immune system upon oral feeding does not depend upon the toxicity of this molecule, but is dependent instead upon some other property shared between it and its B subunit. The experiments described in this paper suggest that this property may be the ability of CT and CTB to bind to ganglioside-like molecules located on the surface of the cells of the villous epithelium. Pierce and coworkers (33, 34) did not examine the ability of galactose or its analogues to inhibit the ability of CT to prime the immune system upon oral administration.

The results presented in this paper demonstrate that it is possible to stimulate the systemic and mucosal immune systems effectively by feeding low doses of certain classes of proteins. These proteins all share the property of being able to
bind specifically to various glycolipids and glycoproteins located on the surface of the cells of the intestinal mucosa and somehow to trigger these cells to internalize the antigens and to transport them to the circulation. The uptake of these molecules can be specifically inhibited by a number of sugars. It is conceivable that viral or bacterial pathogens may use this method of attachment to adhere to and to gain entry into various cells of the gastrointestinal epithelium. The possibility also exists that these mucosal immunogens can be used as carrier proteins to transport other antigens, drugs or hormones more effectively from the intestine into the systemic circulation. These possibilities are currently under study.

Summary

Oral immunization of an animal is generally hard to achieve unless large quantities of antigen are administered. In this study a number of antigens were tested for their ability to elicit a systemic immune response upon oral administration. It was found that bacterial pili, LTB, lectins, and a viral hemagglutinin were all able to elicit significant antibody titers upon oral feeding. The immune response thus generated to LTB and K99 pili could be completely abolished by cofeeding a number of sugars that have close structural homology to the terminal sugars of the GM1 and GM2 gangliosides to which these molecules are known to bind. All of the proteins that were active in oral immunization are known to possess “lectin or lectin-like” binding activities. It is therefore proposed that these molecules are able to bind to glycolipids and glycoproteins on the intestinal mucosa and to stimulate these cells to transport the proteins into the systemic circulation, thereby eliciting a systemic immune response. Molecules that did not possess this binding activity were unable to elicit significant responses at the doses tested.

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