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Expression of a dietary protein in *E. coli* renders it strongly antigenic to gut lymphoid tissue

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SUMMARY

Bacteria that colonize the intestinal mucosa elicit a strong mucosal immune response, whereas food antigens such as ovalbumin are very weakly immunogenic to the gut-associated lymphoid tissue. This may either be due to special physico-chemical properties of bacterial substances versus proteins from animals and plants, or to stimulating properties of the bacteria on, e.g., antigen presentation, rendering all substances contained within bacteria antigenic. To test these hypotheses, ovalbumin was expressed in wild-type *Escherichia coli* and germ-free female rats were colonized with this strain. The systemic and mucosal antibody response of these rats was compared with that of rats given large amounts of dietary ovalbumin. Biliary IgA antibodies, which reflect the local IgA antibody production in the intestine, were only found in the rats colonized with ovalbumin-synthesizing *E. coli*. IgG antibodies in the bile were also only seen in these rats. We conclude that mucosal immunogenicity depends on the context in which a protein is presented to the gut-associated lymphoid tissue, rather than to special antigenic characteristics of the protein in itself.

INTRODUCTION

The gut-associated lymphoid tissue[1,2] is confronted with a wide range of antigenic material: simple food proteins, material from degraded commensal bacteria, and intact micro-organisms brought into contact with the lymphoid tissue by invasion[3] or by translocation.[4] Bacteria colonizing the intestine induce strong mucosal immunity, provided there is good contact with the lymphoid tissue of the gut.[3,5,6] Killed bacteria are also efficient antigens when injected directly into the Peyer's patches.[7] In contrast, dietary antigens, such as ovalbumin, which to a small extent are absorbed antigenically intact[8–10] tend to be very weakly antigenic to the mucosa-associated lymphoid tissue,[11,12] and mainly stimulate systemic immunity.[13,14] This is not due to a lesser degree of absorption of antigenically intact ovalbumin, since this weak antigenicity is also seen upon direct injection of ovalbumin into the Peyer's patches.[12]

There are, thus, indications that the gut-associated lymphoid tissue (GALT) discriminates between dietary proteins and microbial antigens. Such a discrimination can occur by two principally different mechanisms. (i) Bacterial antigens may be intrinsically strongly antigenic due to some physico-chemical properties that distinguish them from, e.g., mammalian proteins of the kind encountered in the diet. Also, there may be an enrichment of B and/or T cells in the GALT which have specificity against bacterial products, such as fimbriae or lipopolysaccharide (LPS) that may be present on the members of the normal gut flora. (ii) Any substance presented to the GALT in a 'bacterial context' may be regarded by the gut-associated lymphoid tissue as strongly antigenic, due to, e.g., stimulation of antigen processing by microbial products. To test these two hypotheses, a plasmid coding for the synthesis of ovalbumin was introduced into a wild-type *Escherichia coli* strain. Germ-free female rats were colonized with the bacteria, and the antibody response against ovalbumin in serum, bile, and milk was recorded. The response was compared with that of rats fed approximately 1 g ovalbumin per day as a dietary constituent.[12]

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MATERIALS AND METHODS

Expression of ovalbumin in E. coli

The plasmid pOMP21 was obtained by fusing cDNA for ovalbumin to the *lac*-operon.[15,16] In the protein synthesized, the first seven amino acids derive from beta-galactosidase; the seventh residue is connected to the fifth residue of ovalbumin. The localization of the ovalbumin when the plasmid is expressed in *E. coli* K-12 is periplasmic and cytoplasmic, but the protein is not secreted.[17] The pOMP21 plasmid was introduced into *E. coli* strain O6K13H1 (Culture Collection of University of Göteborg, Sweden, no. 20561).

Animals

Eleven female rats of the AGUS strain bred in germ-free isolators were monocolonized with *E. coli* O6K13H1 transformed with the pOMP21 plasmid encoding for ovalbumin

production. The rats were kept monocolonized for 16 weeks and ampicillin (0·5 g/l) was included in their drinking water in order for the bacteria to retain the plasmid. During this time they went through two pregnancies and lactation periods. A second group of eight control rats was fed pellets based on egg and milk whey powder during 16 weeks, corresponding to an intake of approximately 1 g ovalbumin per animal and day. They had been monocolonized with the wild-type *E.coli* O6K13H1 before their first pregnancy and reared conventionally during their second pregnancy and lactation period. These rats retained the *E. coli* O6K13H1 in the small intestine and in the colon during the whole time in the conventional environment and their antibody response against ovalbumin and *E. coli* antigens has been described elsewhere.[12] Animals colonized with the wild-type *E. coli* but not fed ovalbumin do not develop any antibodies against ovalbumin.

Collection of samples

Bacterial growth was recorded from different levels of the gastrointestinal tract by cultivation on Drigalski agar and on tryptic soy agar containing ampicillin. To assess expression of type 1 fimbriae, three to five bacterial 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, and designated mannose-sensitive haemagglutination and taken as an indication of type 1 fimbriation.[18]

Milk, bile and serum were sampled from ether-anaesthetized rats on Day 12 of the lactation period and assayed for antibodies against ovalbumin.

Antibody determinations

Polyvinyl microtitre plates (MIC-2000; Dynatech, Alexandria, VA) were coated with 10 μg/ml of ovalbumin (Sigma, St Louis, MO) in PBS. The samples were diluted from 1/10 to 1/10 000 in PBS with 0·05% Tween 20 (Kebo, Stockholm, Sweden) and incubated for 4 hr. After washing, alkaline phosphatase-conjugated anti-rat IgG (Cappel, West Chester, PA), anti-rat IgA (Nordic, Tilburg, The Netherlands) or IgM (affinity-purified; Cappel) was added and incubated over night. Para-nitrophenylphosphate (1g/l) in diethanolamine buffer (1 M, pH 9·8) was used as substrate and the absorbance at 405 nm was recorded at 100 min (Titertec Multiscan, Flow Labs, MacLean, VA). The titres of the individual samples were calculated as the inverted value of the dilution giving an absorbance of 0·1 over the background. The background was milk, bile or serum samples from germ-free AGUS rats.

Quantification of total immunoglobulin levels

This was done with a sandwich ELISA where all steps were similar to the above-mentioned ELISA technique except for the following. Polystyrene microtitre plates were coated overnight at room temperature, with heavy chain-specific affinity-purified anti-rat IgM, anti-rat IgA, or anti-rat IgG at 2 mg/ml (Bethyl Laboratories Inc., Montgomery, TX). Any immunoglobulin in the sample bound to the solid phase was detected with peroxidase-labelled monoclonal anti-rat IgM, anti-rat IgA (Zymed Laboratories Inc, San Francisco, CA) or affinity-purified γ-chain-specific anti-rat IgG (Southern Biotechnology, Birmingham, AL). Azinoethylbenzthiazoline-sulphonic acid was used as substrate (Sigma). The absorbance obtained from appropriately diluted samples was compared with the absorbance values obtained from a standard curve produced with known concentrations of pure myeloma IgM or IgA protein (PharMingen, San Diego, CA). The absorbance with the anti-IgG conjugate was compared to a standard curve consisting of different dilutions of a normal rat serum. Thus the level of IgG could only be expressed as relative levels. The lower limit of detection of this assay was 1 ng/ml.

Statistics

The antibody response was compared between rats colonized with *E. coli* pOMP21 and rats receiving diet containing ovalbumin by Wilcoxon's rank sum test.

RESULTS

The bacterial colonization pattern of the rats monocolonized with the gene-manipulated *E. coli* is shown in Table 1. The rats all carried the bacteria in caecum and colon during the whole study period. In some rats bacteria could also be recovered from lower ileum and other parts of the gastro-intestinal tract. A few rats also showed growth from within the Peyer's patches, indicating a translocation of viable bacteria over the epithelium. The recovered bacteria expressed type 1 fimbriae, as evidenced by a mannose-sensitive agglutination of guinea-pig erythrocytes after one subculture on Drigalski agar.

The antibody responses against ovalbumin are shown in Fig. 1. Whereas both modes of delivery of ovalbumin could induce moderate titres of serum and milk antibodies, only ovalbumin presented to the gut as a bacterial antigen induced high levels of IgA or IgG antibodies in the bile.

The concentration of total immunoglobulins (IgA, IgG and IgM) in the sera did not differ between the two groups of rats and the levels were similar to the levels found in conventional rats (not shown).

DISCUSSION

In the rat, biliary IgA results from a transfer of dimeric IgA from serum after coupling to secretory component on the hepatocytes.[19,20] The dimeric IgA transferred into the bile is mainly produced in the intestinal lamina propria.[7,21] In this species, the major route of secretion of IgA into the lumen of the intestine is, in fact, via the bile, which accounts for 90% of the intestinal IgA,[22] whereas in man the direct transfer of dimeric IgA over the intestinal epithelium predominates.[23] In the rat, IgA antibodies in the bile are, thus, representative of the local IgA production in the intestine. We have never been able to get an IgA antibody response against ovalbumin, even with feeding two different diets with high or low concentrations of ovalbumin for up to 4 months or injecting ovalbumin into the Peyer's patches. Others have also shown that feeding small amounts of ovalbumin for several days to rats does not result in any production of antibodies.[24] Thus, the finding of biliary IgA antibodies against ovalbumin in all rats colonized with ovalbumin-synthesizing *E. coli*, but not in any of the rats fed ovalbumin in their diet, indicates that only ovalbumin presented to the gut immune system within bacteria, although presumably in a very low concentration, has the capacity to evoke an intestinal IgA response.

Table 1. Pattern of gastrointestinal colonization by ampicillin-resistant *E. coli*

	Growth of *E. coli* O6K13							
Rat no.	mou	sto	duo	jej	ile	Pp	cae	col
1	+	−	−	−	++	−	+++	+++
2	+	−	−	+	+	−	+++	−
3	+	−	−	−	+	−	+++	+++
4	+	−	−	−	++	−	+++	+++
5	+	+++	−	++	+++	−	+++	+++
6	+	+++	−	+++	+++	−	+++	+
7	++	+	+	++	+++	−	+++	+++
8	++	+++	++	−	+++	−	+++	+++
9	++	+	−	+++	+++	+	+++	+++
10	+	++	−	+	++	+++	+++	+++
11	+	+	−	−	+++	+	+++	+++

Gut contents were sampled with a cotton-tipped swab at killing, and cultivated on ampicillin-containing tryptic soy agar plates. The growth after overnight aerobic culture was recorded as follows: +++ denotes heavy growth, ++ moderate growth, and + growth of a few colonies per plate. Abbreviations: mou, mouth; sto, stomach; duo, duodenum; jej, jejunum; ile, ileum; Pp, interior of Peyer's patches; cae, caecum; col, colon.

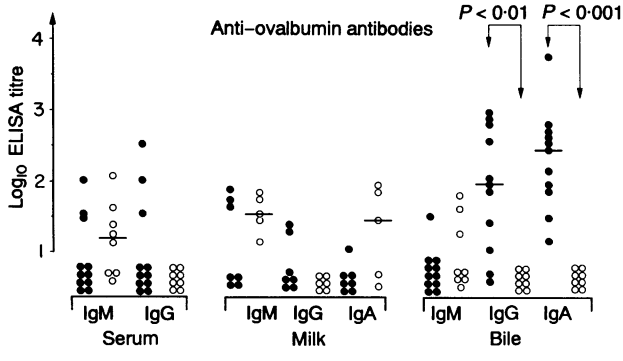

Figure 1. Antibodies in bile, serum and milk in rats colonized with *E. coli* carrying the plasmid pOMP21 encoding ovalbumin synthesis (filled circles), or in rats fed approximately 1 gram per day of ovalbumin in the diet (open circles). Data from the ovalbumin-fed group have been published previously.[12] The ELISA antibody titres were defined as the reciprocal of the dilution giving an absorbance of 0·100 over a background sample of bile, serum or milk from germ-free rats. A titre of < 1, thus, means that the absorbance of the 1/10 dilution was not higher than 0·100 over that from unimmunized animals. Each symbol represents one animal and the bar denotes the median. Significance testing was performed with the Wilcoxon rank sum test and the *P* values for the indicated differences are shown in the figure.

Ovalbumin synthesized by *E. coli* carrying the pOMP21 plasmid localizes in the cytoplasm, and in the periplasmic space, but is not secreted.[17] It can thus only be found extracellularly if the bacteria die and decompose. Since the total mass of intestinal bacteria is approximately 1 g (dry weight) in the rat, at the most milligram quantities of ovalbumin can have been present in the *E. coli* pOMP21-colonized rats, whereas the control group was fed approximately 1 g per day of pure ovalbumin. Since whole bacteria are much less likely to pass over the intestinal epithelium than protein molecules[12] it can safely be assumed that the ovalbumin diet group, colonized with the wild type *E. coli*, encountered lots more ovalbumin than the *E. coli* pOMP21-colonized group.

Although the mammary gland belongs to the common mucosal immune system, not only cells primed in the gut-associated lymphoid tissue are trapped in the lactating mammary gland, but also cells involved in systemic antibody responses.[25] The mammary gland also deviates from, e.g., the intestinal lamina propria in that the environment favours proliferation and maturation of blasts.[25] The mammary gland thus expands systemic as well as mucosal ongoing immune responses, and the antibodies found in milk may therefore reflect either systemic or mucosal immunity, or a combination thereof.

The mucosal antigenicity given to ovalbumin when expressed in bacteria, indicates that dietary antigens do not possess special antigenic characteristics, but that instead antigenicity is conferred on the protein because it is encountered by the gut-associated lymphoid tissue within bacteria. It was not sufficient to feed the rats ovalbumin and colonize them with the wild-type *E. coli*, even if these animals had substantial levels of ovalbumin in the circulation. This indicates that the dendritic macrophages of the gut, which are believed to act as antigen presenters,[26,27] take up intact bacteria and present all the contained proteins to lymphocytes in a stimulatory fashion. Proteins encountered in solution would therefore not stimulate immunogenic antigen presentation by these cells. The process of phagocytosis may stimulate macrophages to antigen presentation. In addition, bacterial LPS and lipoproteins increase IL-1 and IL-6 production by macrophages.[28,29] The elaboration of these interleukins when antigen is presented may be necessary to stimulate T cells.[30] In particular Th2 cells, which stimulate IgA production, seem to depend on stimulation by IL-1 to be activated by antigen-presenting cells.[31,32]

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REFERENCES

1. Hanson L.Å. & Brandtzaeg P. (1989). The mucosal defence system. In: *Immunological Diseases in Infants and Children* (ed. R.T. Stiehm), 3rd edn, p. 116. Saunders, Philadelphia.
2. Mestecky J. (1987) The common mucosal immune system and current strategies for induction of immune responses in external secretions. *J. clin. Immunol.* **7,** 265.
3. Hohmann A., Schmidt G. & Rowley D. (1979) Intestinal and serum antibody responses in mice after oral immunization with *Salmonella, Escherichia coli* and *Salmonella-Escherichia coli* hybrid strains. *Infect. Immun.* **25,** 27.
4. Berg R.D. (1983). Translocation of indigenous bacteria from the intestinal tract. In: *Human Intestinal Microflora in Health and Disease* (ed. D. J. Hentges), p. 333. Academic Press, New York.
5. Pierce P., Kaper J., Mekalanos J., Cray W. & Richardsson K. (1987) Determinants of the immunogenicity of live virulent and mutant *Vibrio cholerae* in rabbit intestine. *Infect. Immun.* **55,** 477.
6. Fubara E.S. & Freter R. (1972) Source and protective function of coproantibodies in intestinal disease. *Am. J. clin. Nutr.* **25,** 1357.
7. Dahlgren U., Ahlstedt S., Andersson T., Hedman L. & Hanson L.Å. (1983) IgA antibodies in rat bile are not solely derived from thoracic duct lymph. *Scand. J. Immunol.* **17,** 569.
8. Kilshaw P.J. & Cant A.J. (1985) The passage of maternal dietary antigen into human breast milk. *Int. Arch. Allergy appl. Immunol.* **75,** 8.
9. Husby S., Jensenius J.C. & Svehag S.E. (1985) Passage of undegraded dietary antigen into the blood of healthy adults. Quantification, estimation of size distribution and relation of uptake to levels of specific antibodies. *Scand. J. Immunol.* **22,** 83.
10. Walker W.A. & Isselbacher K.J. (1974) Uptake and transport of macromolecules by the intestine. Possible role in clinical disorders. *Gastroenterology,* **67,** 531.
11. Peri B.A., Theodore C.M., Losonsky G.A., Fishaut J.M., Rothberg R.M. & Ogra P.L. (1982) Antibody content of rabbit milk and serum following inhalation or ingestion of respiratory syncytial virus and bovine serum albumin. *Clin. exp. Immunol.* **48,** 91.
12. Wold A., Dahlgren U., Hanson L., Mattsby-Baltzer I. & Midtvetdt T. (1989) Difference between bacterial and food antigens in mucosal immunogenicity. *Infect. Immun.* **57,** 2666.
13. Husby S., Jensenius J.C. & Svehag S.E. (1985) ELISA quantitation of IgG subclass antibodies to dietary antigens. *J. immunol Meth.* **82,** 321.
14. Russel M., Hammond D., Radl J., Haaijman H. & Mestecky J. (1985) Secretory IgA1 and IgA2 responses to environmental antigens. *Prot. Biol. Fluids.* **152,** 77.
15. Mercereau-Puijalon O. & Kourilsky P. (1979) Introns in the chicken ovalbumin gene prevent ovalbumin synthesis in *E. coli* K-12. *Nature,* **279,** 647.
16. Mercereau-Puijalon O., Royal A., Cami B., Garapin A., Krust A., Gannon F. & Kourilsky P. (1978) Synthesis of an ovalbumin-like protein by *Escherichia coli* K-12 harbouring a recombinant plasmid. *Nature,* **275,** 505.
17. Baty D., Mercereau P.O., Perrin D., Kourilsky P. & Lazdunski C. (1981) Secretion into the bacterial periplasmic space of chicken ovalbumin synthesized in *Escherichia coli. Gene,* **16,** 79.
18. Duguid J.P. & Gillis R.R. (1957) Fimbriae and adhesive properties in dysenteric bacilli. *J. Pathol. Bacteriol.* **74,** 397.
19. Fisher M.M., Nagy B., Bazin H. & Underdown B.J. (1979) Biliary transport of IgA: role of secretory component. *Proc. natl. Acad. Sci. U.S.A.* **76,** 2008.
20. Orlans E., Peppard J., Reynolds J. & Hall J. (1978) Rapid active transport of immunoglobulin A from blood to bile. *J. exp. Med.* **147,** 588.
21. Hall J. (1978) Lymphatic physiology and secretory immunity. *Adv. exp. Med. Biol.* **107,** 29.
22. Lemaitre-Coelho I., Jackson G.D.F. & Vaerman J.P. (1978) Relevance of biliary IgA antibodies in rat intestinal immunity. *Scand. J. Immunol.* **8,** 459.
23. Jonard P.P., Rambaud J.C., Dive C., Vaerman J.P., Gabian A. & Delacroix D.L. (1984) Secretion of immunoglobulins and plasma proteins from the jejunal mucosa. Transport rate and origins of polymeric immunoglobulin A. *J. clin. Invest.* **74,** 525.
24. Ahlstedt S. & Björkstén B. (1983) Specific antibody responses in rats and mice after daily immunization without adjuvant. *Int. Archs. Allergy appl. Immunol.* **71,** 293.
25. Parmely M.J. & Manning L.S. (1983) Cellular determinants of mammary cell-mediated immunity in the rat: kinetics of lymphocyte subset accumulation in the rat mammary gland during pregnancy and lactation. *Ann. NY Acad. Sci.* **409,** 517.
26. Mayrhofer G., Pugh C.W. & Barclay A.N. (1983) The distribution, ontogeny and origin of the rat Ia-positive cells with dendritic morphology and of Ia antigen in epithelia with special reference to the intestine. *Eur. J. Immunol.* **13,** 112.
27. Mahida Y.R., Yu K.C. & Jewell J.P. (1988) Characterization of antigen presenting activity of intestinal mononuclear cells isolated from normal and inflammatory bowel disease colon and ileum. *Immunology,* **65,** 543.
28. Coulle P.G., Cayphes S., Vink A., Uyttenhove C. & Van Snick J. (1987) Inteleukin HP-1-related hybridoma and plasmacytoma growth factors induced by lipopolysaccharide *in vivo. Eur. J. Immunol.* **17,** 1217.
29. Dinarello C.A. (1984) Interleukin-1. *Rev. Inf. Dis.* **6,** 51.
30. McKenzie D. (1988) Alloantigen presentation by B cells. Requirement for IL-1 and IL-6. *J. Immunol.* **141,** 2907.
31. Kurt-Jones E.A., Hamberg S., Ohara J., Paul W.E. & Abbas A.K. (1987) Heterogeneity of helper/inducer T lymphocytes. I. Lymphokine production and lymphokine responsiveness. *J. exp. Med.* **166,** 1774.
32. Coffman R.L. & Mosmann T.R. (1988) Isotype regulation by helper T cells and lymphokine. *Monogr. Allergy,* **24,** 96.