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Induction of oral tolerance in rats without Peyer's patches

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SUMMARY

The induction of oral tolerance after ingestion of antigen has been reported in several animal models. The precise mechanisms responsible for this unresponsiveness are not well understood. As some investigations have suggested a key role of Peyer's patches suppressor T cells, an animal model was developed in which the PP were surgically removed. Using this model, the influence of the PP on the induction of oral tolerance against SRBC was investigated. In order to induce tolerance, the rats were fed SRBC on four consecutive days. On Day 5 they were i.p. challenged by injection of SRBC, and 5 days later the number of immunoglobulin-secreting cells against SRBC was determined within the spleen. Using this protocol, the oral tolerance induction could be shown very clearly in control animals as well as in rats without PP. Therefore, tolerance induction is possible in the absence of PP-T cells. Other mechanisms must be responsible for the tolerance induction in this model.

INTRODUCTION

The phenomenon of immune tolerance after feeding of defined antigens was reported as early as 1911 by Wells, who demonstrated that guinea-pigs lost the ability to develop systemic anaphylaxis after ingestion of ovalbumin (Wells, 1911). In 1946 Chase demonstrated that oral administration of dinitrochlorobenzene inhibits the delayed-type hypersensitivity to this antigen (Chase, 1946). The existence of antigen-specific systemic unresponsiveness after feeding or intragastric intubation of antigens has long been recognized (reviewed in Kagnoff, 1981; Tomasi, 1980), but the precise mechanisms responsible for this unresponsiveness are not well understood. Several mechanisms are subject of discussion. Suppressor T cells have been documented in unresponsive mice after the feeding of sheep erythrocytes (Mattingly & Waksmann, 1978; MacDonald, 1983) and of ovalbumin (Richman *et al.*, 1978, Ngan and Kind, 1978). These suppressor cells can be detected very soon after gastric inoculation in the PP and the mesenteric lymph nodes before they arrive in the spleen or other lymphatic structures, suggesting that migration of suppressor cells may occur.

However, unresponsiveness can be established by oral administration of antigen in the absence of suppressor cells, as demonstrated by several investigators. In these experiments, suppression has long been transferred from gastrically inoculated animals to normal animals by serum factors possibly containing antigen–antibody complexes (Andre *et al.*, 1975; Chalon, Milne & Vaerman, 1979) or antiidiotypic antibodies (Kagnoff, 1978), which induce negative-feedback effects. Furthermore, this antibody-mediated suppression can be transferred by B cells acting as suppressor B cells (Asherson *et al.*, 1977) or inducing secondary suppressor T cells (L'age Stehr *et al.*, 1980). Nearly all these suggested mechanisms have in common the fact that the PP play a key role for the induction of oral tolerance. One reason is that the epithelium overlying PP contains specialized M cells (Owen, 1977), which are able to ingest macromolecules (Keljo & Hamilton, 1983) and bring antigens into direct contact with lymphoid tissue in the PP. Thus, enteric antigens stimulate the proliferation of PP cells *in situ* (Cebra *et al.*, 1979, Gearhart & Cebra, 1979), producing for example suppressor cells which are later found in the spleen.

In all these investigations, more or less indirect experimental designs have been used to explain the significance of PP in the induction of oral tolerance.

In this paper, the direct influence of PP on the induction of oral tolerance was investigated using an animal model in which rats were deprived of their PP. These animals were fed sheep erythrocytes and their reaction to an i.p. challenge was tested afterwards.

Abbreviations: ISC, Ig-secreting cells; M cells, microfold cells; PBS, phosphate-buffered saline; PFC, plaque-forming cells; PP, Peyer's patches; SRBC, sheep red blood cells.

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MATERIALS AND METHODS

Animals

Conventional Wistar rats of both sexes raised at the laboratory were used. They had an average weight of 250 g. They received water *ad libitum* and were fed a conventional rat diet.

Antigens

Sheep red blood cells obtained from our own Institute were washed three times with PBS before being used.

Immunization

Intragastric immunization was performed by giving 4×10^9 sheep red blood cells (SRBC) by intragastric intubation on four

consecutive days. Challenge consisted of a single i.p. injection of 2×10^9 SRBC on Day 5.

Surgical procedure

Under chloralhydrate anaesthesia, the PP were removed by laying a U-shaped suture around the patch and cutting it off. This wound was then covered by a serosal suture. After operation, the rats were fed only acidified water for three consecutive days. Experiments were initiated 4 weeks afterwards. Controls were sham-operated by removing small parts of the bowel.

Haemolytic plaque assay

PFC were assayed by a modification of the Jerne plaque assay using guinea-pig complement to lyse antibody-coated cells (Jerne & Nordin, 1968). Indirect plaques were developed with rabbit anti-rat IgG (Dakopatts, Hamburg, FRG) or goat anti-rat IgA (Miles Scientific, Munich, FRG). Each measurement was done in triplicate. In order to prove specificity of priming and/or tolerance induction, the plaque assay was also performed using horse red blood cells in all tests. This background, which never exceeded 10 PFC/10^6 cells, was subtracted.

Immunofluorescence

In order to determine the T-cell subpopulations in mesenteric lymph nodes and spleen, an indirect immunofluorescence method was used. Spleen cells were separated by a Ficoll gradient and incubated with monoclonal mouse anti-rat antisera specific for T-helper cells (W 3–25), T cells (W 3–13) and Ia (Ox 12, Serotec Ltd, Bicester, Oxon, U.K.). After incubation at 4° and several washings, the cells were incubated with a fluorescence-labelled anti-mouse immunoglobulin (Hybritech Europe, Liège, Belgium) which had been absorbed with rat spleen cells.

Statistical analysis

The Wilcoxon test for odd number was used.

RESULTS

Control of the PP removal

The completeness of the PP removal was checked by light and electron microscopy at weekly intervals up to 12 weeks. The place of the excision, all other suspicious-looking parts of the intestine and some randomly taken gut segments were examined for the regeneration of follicles. In accordance with older reports from the literature (Sanders & Florey, 1940), we could not find any lymphoid follicle-like structure.

Splenic PFC in control rats and rats without PP

Sham-operated rats, unoperated rats and rats without PP were injected intraperitoneally with SRBC. Five days later, the animals were killed and the number of anti-SRBC plaque-forming cells determined within the spleen and the mesenteric lymph nodes. Figure 1 shows the distribution of immunoglobulin-secreting cells against SRBC after intraperitoneal immunization of control rats and rats without PP. Control rats had, on average, 250 IgG-, 500 IgA- and 450 IgM-producing cells per 10^6 spleen cells. The relatively low number of IgG-producing cells is the result of the 5-day interval between immunization and test.

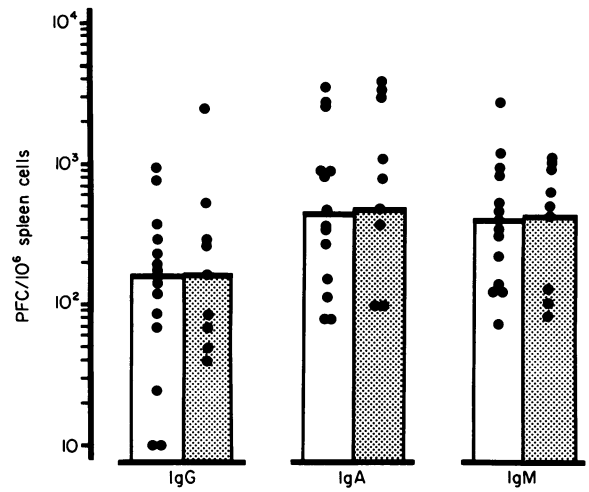

Figure 1. PFC with specificity for SRBC in the spleen after i.p. immunization. The results are the medians of sham-operated control animals (open bars, $n = 15$) and PP-deprived rats (dotted bars, $n = 10$). The number of PFCs is plotted on a logarithmic scale.

Interestingly, the IgA response is as high as the IgM response. In rats without PP, the i.p. immunization yields comparable PFC responses in the spleen. Five days after immunization, the IgG and IgM response was comparable with the results obtained in control animals. The plaque-forming cell responses for SRBC in the mesenteric lymph nodes were very low, but comparable between control rats and rats without PP.

Induction of antigen-specific unresponsiveness by intragastric intubation of SRBC in control animals

After intragastric administration of SRBC for four consecutive days, the animals were challenged by SRBC injected intraperitoneally. Five days later, the spleen and the mesenteric lymph nodes were removed and investigated for SRBC-reactive cells using a haemolytic plaque assay. As shown in Fig. 2, a

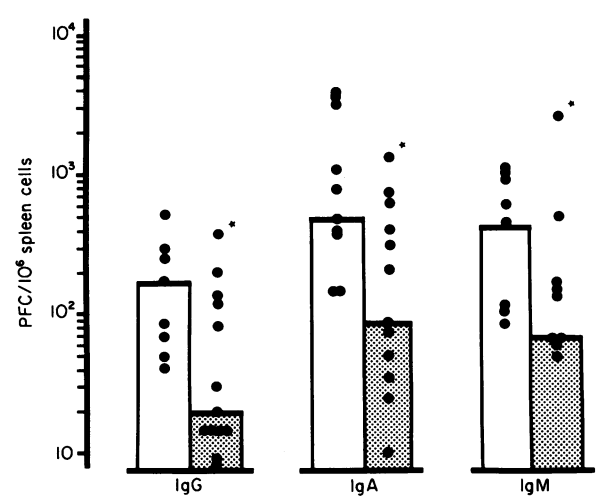

Figure 2. Oral tolerance induction in sham-operated control animals. PFC after i.p. immunization (open bars, $n = 15$) and after AG feeding and i.p. challenge (dotted bars, $n = 15$). The difference between the groups is statistically significant ($P < 0.05$) for all three isotypes.

diminished response was found after oral ingestion of SRBC. This unresponsive state could be shown for all three immunoglobulin classes investigated. The results obtained were statistically significant. The lymphocytes of the mesenteric lymph nodes contained few plaque-forming cells after i.p. injection alone or after oral tolerance induction.

Induction of antigen-specific unresponsiveness by intragastric intubation of SRBC in PP-deprived rats

Four weeks after removal of the PP, the animals were fed SRBC by intragastric intubation on 4 days. One day later they were challenged intraperitoneally with SRBC, and 5 days afterwards the number of cells secreting anti-SRBC was determined within the mesenteric lymph nodes and the spleen. Whereas the response in mesenteric lymph nodes was not significantly different (probably the result of the very low response), the number of PFCs within the spleen showed a sharp decrease after oral feeding of rats with SRBC. Contrary to the commonly accepted role of PP for the induction of oral tolerance in rats lacking PP, antigen feeding also resulted in significantly diminished responsiveness. As in control animals, this was evident in respect of all three immunoglobulin types. The results are given in Fig. 3. The comparison of the results obtained after tolerance induction in control rats and rats without PP is given in Fig. 4. The results clearly indicated that the tolerance induction was not significantly affected by removal of the PP. This applied to IgG, IgA and IgM responses.

Distribution of T cells in rats without PP and control animals

Using monoclonal antibodies, the T-cell subpopulations of the spleen were counted. There was no significant difference for all T cells or T helper cells, either between sham-operated and PP-deprived animals, or between immunized and tolerized animals. The T helper/T ratio was between $62 \pm 20\%$ and $50 + 20\%$.

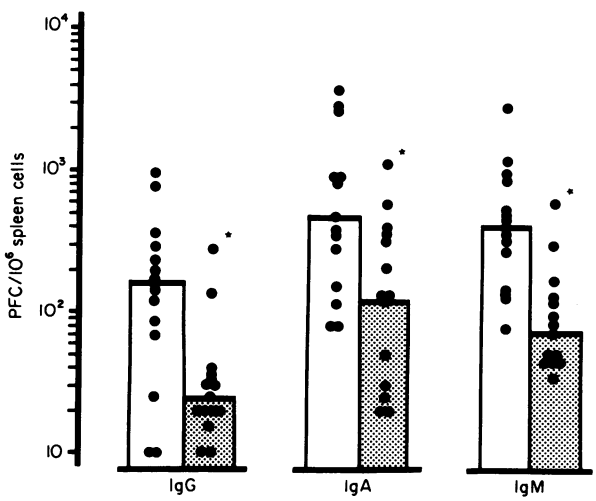

Figure 3. Oral tolerance induction in PP-deprived rats. PFC after i.p. immunization (open bars, $n = 15$) and after SG feeding and i.p. challenge (dotted bars, $n = 14$). The difference is significant ($P < 0{\cdot}05$).

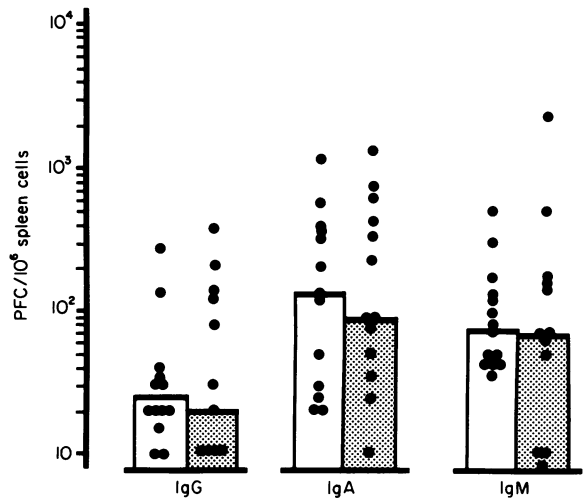

Figure 4. Oral tolerance induction in sham-operated controls (open bars, $n = 15$) and PP-deprived rats (dotted bars, $n = 14$).

DISCUSSION

The present study confirms the fact that antigen-specific immunological unresponsiveness can be established to SRBC by the intragastric administration of this antigen. A diminished response after oral intubation was seen in the spleen for all three antibody classes examined. Earlier investigations have indicated that the PP play a key role in the induction of oral tolerance. In order to examine their influence on the induction of tolerance more precisely, we developed an animal model in which all PP were surgically removed. Normally 20–30 plaques must be cut off along the whole intestine of the rat. In accordance with reports from the literature, the histological examination of the intestine did not show any new formation after removal (Sanders & Florey, 1940; Enders *et al.*, 1980; Enders & Seifert, 1983). As the operation may cause an alteration of the animal's reactivity to an AG-challenge, the response to i.p. applicated SRBC was tested initially. Four weeks after operation, the animals responded to an i.p. administered antigen as well as control animals. This was essential to the following experiment of the induction of hyporesponsiveness. After feeding SRBC and i.p. challenge, sham-operated as well as PP-derived rats had very low-SRBC responses in the spleen.

This result brings up the question of antigen-handling in the gut. In this respect, the investigation had centred on the role of the small intestinal PP since they possess some prerequisites for a potent immune response: AG-delivery via a specialized epithelium (M cells; Owen & Jones, 1974), macrophages, T- and B-cell regions (Witmer & Steinman, 1984). As PP-deprived animals lacking all these cells have a comparable oral tolerance induction as controls, other AG-handling mechanisms must be discussed. In this context, some authors have pointed out the importance of small lymph nodules scattered along the intestine (Keren *et al.*, 1978; Hamilton *et al.*, 1981). These lymph nodules, however, have been described in rabbits, and the existence of functional M cells was concluded from the rareness of goblet cells in the epithelium of these nodules in analogy to PP. Another explanation for the adequate AG-handling in the gut without PP is the observation that intestinal epithelial cells are able to express Class II MHC antigens (Curman *et al.*, 1979;

Mayrhofer, Pugh & Barclay, 1983), which have been shown to be important in the presentation of antigen to T cells. These T cells are distributed along the subepithelial spaces, and may play a regulatory role for the Ia expression of the epithelium (Cerf-Bensussan *et al.*, 1984). In this way, epithelial cells can directly present the AG to immunocompetent T cells, which exert all the functions discussed for the induction of oral tolerance like T-suppressor cells or regulated B cells.

The demonstration that intragastric immunization may lead to systemic hyporesponsiveness, even in the absence of the PP, raises speculation on the function of the epithelium, the intraepithelial lymphocytes and the T lymphocytes of the lamina propria in the regulation of an immune response to an orally applied antigen and for the regulatory cells responsible for the isotype switch. *In vitro* experiments using separated T lymphocytes will give further insight into the interaction of the cells of the gut for an immune response.

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REFERENCES

ANDRE C., HEREMANS J.F., VAERMAN J.P. & CAMBIASO C.L. (1975) A mechanism for the induction of immunological tolerance by antigen feeding: antigen antibody complexes. *J. exp. Med.* **142,** 1509.

ASHERSON G.L., ZEMBALA M., PERERA M.A.C.C., MAYHEW B. & THOMAS W.R. (1977) Production of immunity and unresponsiveness in the mouse by feeding contact sensitizing agents and the role of suppressor cells in the Peyer's patches, mesenteric lymph nodes and other lymphoid tissue. *Cell. Immunol.* **33,** 145.

CEBRA J.J., CRANDALL C.A., GEARHART P.J., ROBERTSON S.M., TSENG J. & WATSON P.M. (1979) Cellular events concerned with the initiation, expression and control of the mucosal immune response. In: *Immunology of Breast Milk* (eds P. Ogra and D. Danton), p. 1. Raven Press, New York.

CERF-BENSUSSAN N., QUARONI A., KURMICK J.T. & BHAN A.K. (1984) Intraepithelial lymphocytes modulate Ia expression by intestinal epithelial cells. *J. Immunol.* **132,** 2244.

CHALON M.P., MILNE R.W. & VAERMAN J.P. (1979) *In vitro* immunosuppressive effect of serum from orally immunized mice. *Eur. J. Immunol.* **9,** 747.

CHASE M.S. (1946) Inhibition of experimental drug allergy by prior feeding of the sensitizing agent. *Proc. Soc. exp. Biol.* **61,** 257.

CURMAN B., KÄMPE O., RASK L. & PETERSON P.A (1979) Presence of alloreactive Ia antigens on murine intestine epithelial cells. *Scand. J. Immunol.* **10,** 11.

ENDERS G. & SEIFERT J. (1983) Zur Bedeutung der Peyerschen Plaques für die Darmimmunität. *Fortscher. Med.* **101,** 183.

ENDERS G., SEIFERT J., HAUFFE A. & BRENDEL W. (1980) Immune response to an orally applied antigen after removal of the Peyer's patches. *Immunobiology*, **157,** 216.

GEARHART P.J. & CEBRA J.J., (1979) Differentiated B-lymphocytes. Potential to express particular antibody variable and constant regions depends on site of lymphoid tissue and antigen load. *J. exp. Med.* **149,** 216.

HAMILTON S.R., KEREN D.F., YARDLEY J.H. & BROWN G. (1981) No impairment of local intestinal immune response to keyhole limpet haemocyanin in the absence of Peyer's patches. *Immunology*, **42,** 431.

JERNE N.K. & NÖRDIN A.A. (1968) Plaque formation in agar by single antibody-producing cells. *Science*, **140,** 405.

KAGNOFF M.F. (1978) Effects of antigen feeding on intestinal and systemic immune response. III. Antigen specific serum mediated suppression of humoral antibody responses after antigen feeding. *Cell. Immunol.* **40,** 186.

KAGNOFF M.F. (1981) Immunology of the digestive system. In: *Physiology of the Gastrointestinal Tract* (ed. L. R. Johnson), p. 1337. Raven Press, New York.

KELJO D.J. & HAMILTON J.F. (1983) Quantitative determination of macromolecular transport rate across intestinal Peyer's patches. *Am. J. Physiol.* **244,** 637.

KEREN D.F., HOLT P., COLLINS H.H., GEMSKI P. & FORMAL S.B. (1978) The role of Peyer's patches in the local immune response of rabbit ileum to live bacteria. *J. Immunol.* **120,** 1892.

L'AGE STEHR J., TEICHMANN H., GERSHON R. & CANTOR H. (1980) Stimulation of regulatory T-cell circuits by immunoglobulin-dependent structures on activated B-cells. *Eur. J. Immunol* **10,** 21.

MACDONALD T.A. (1983) Immunosuppression caused by antigen feeding. II. Suppressor T-cells mask Peyer's patches B-cells primary to orally administered antigen. *Eur. J. Immunol.* **13,** 138.

MAYRHOFER G., PUGH C.W. & BARCLAY A.G. (1983) The distribution, ontongeny and origin in the rat of Ia positive cells with dendritic morphology and of Ia antigen in epithelia, with special reference to the intestine. *Eur. J. Immunol.* **13,** 112.

MATTINGLY J.A. & WAKSMANN B.H. (1978) Immunologic suppression after oral adminstration of antigen. I. Specific suppressor cells formed in rat Peyer's patches after oral administration of sheep erythrocytes and their systemic migration. *J. Immunol.* **121,** 1878.

NGAN J. & KIND L.A. (1978) Suppressor T-cells for IgE and IgG in Peyer's patches of mice made tolerant by the oral administration of albumin. *J. Immunol.* **120,** 861.

OWEN R.L. (1977) Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine. An ultrastructural study. *Gastroenterology*, **72,** 440.

OWEN R.L. & JONES A.L. (1974) Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. *Gastroenterology*, **63,** 1160.

RICHMAN L.K., CHILLER J.M., BROWN W.R., HANSON D.G. & VAZ M.N. (1978) Enterally induced immunologic tolerance. I. Induction of suppressor T-lymphocytes by intragastric administration of soluble proteins. *J. Immunol.* **121,** 2429.

SANDERS A.G. & FLOREY H.W. (1940) The effects of the removal of lymphoid tissue. *Bri. J. exp. Pathol.* **21,** 275.

TOMASI T.B. (1980) Oral tolerance. *Transplantation*, **29,** 353.

WELLS H.G. (1911) Studies on the chemistry of anaphylaxis. III. Experiments with isolated proteins, especially those of the hens egg. *J. infect. Dis.* **9,** 147.

WITMER M.D. & STEINMAN R.M. (1984) The anatomy of peripheral lymphoid organs with emphasis on accessory cells: light microscopic immunocytochemical studies of mouse spleen, lymph node, and Peyer's patches. *Am. J. Anatomy*, **170,** 465.