Effect of the Gastrointestinal Microflora on Induction and Maintenance of Oral Tolerance to Ovalbumin in C3H/HeJ Mice

MARIE-CHRISTIANE MOREAU* AND GERARD CORTHIER

Laboratoire d'Ecologie Microbienne, CRJJ, INRA, Jouy-en-Josas, 78350, France

The effect of the digestive microflora on oral tolerance to ovalbumin was studied by using axenic (germfree) and conventional C3H/HeJ mice. In contrast to reported results of studies with sheep erythrocytes, oral administration of ovalbumin induced tolerance in axenic mice, but the maintenance of tolerance was found to be of shorter duration than was found with conventional mice. These data indicate that the contribution of the microflora to oral tolerance depends on the antigen used.

The induction of tolerance by oral administration of an antigen prevents digestive hypersensitivity reactions to food antigens. The mechanisms and factors involved in oral tolerance are still poorly understood (reviewed in references 2 and 13). Contrasting results obtained with two antigens, ovalbumin (OVA) and sheep erythrocytes (SRBC), have been published, and it is uncertain whether the same mechanisms are involved in oral tolerance to SRBC as in tolerance to OVA. Kiyono et al. (6) could not induce oral tolerance to SRBC in lipopolysaccharide (LPS)-nonresponsive C3H/HeJ mice, while several authors showed that this strain of mice could be rendered tolerant to OVA by prior oral feeding (14, 18). Furthermore, by using SRBC, it has been established that the gastrointestinal microflora is an important factor for induction of oral tolerance, since BALB/c axenic mice could not be made tolerant to orally administered SRBC, in contrast to conventional BALB/c mice, which were able to develop tolerance to SRBC (23). The present study evaluates the role of the gastrointestinal microflora in the induction and maintenance of the OVA oral tolerance during a 3-month period after OVA feeding.

Axenic and conventional C3H/HeJ male and female mice, 2 months old, were obtained from the Centre National de la Recherche Scientifique, Orleans-la-Source, France. They were LPS unresponsive. Axenic mice were maintained in Trexler plastic isolators.

Groups of mice (10 each) were made tolerant ("tolerized") by gastric intubation of 20 mg of OVA (grade V; Sigma Chemical Co.) dissolved in 0.5 ml of 0.15 M NaCl. A control group was fed a similar volume of saline. Mice were immunized intraperitoneally (i.p.) with 250 μg of OVA emulsified in complete Freund adjuvant 8 and 21 days (priming and booster i.p. injections) after the oral feeding. Blood samples were collected from the retro-orbital plexus either 28 days or 33, 42, and 54 days after OVA feeding. A third challenge systemic immunization was realized by intramuscular injection of 100 μg of OVA without complete Freund adjuvant, 62 days after feeding. Mice were again bled 15 and 28 days after the final challenge (77 and 90 days after the initial oral dose).

Anti-OVA immunoglobulin G (IgG) levels were measured by an enzyme-linked immunosorbent assay. Briefly, Linbro microplates were coated with OVA (10 μg/ml; Sigma, grade VI) in carbonate buffer, pH 9.6, and incubated overnight at 4°C. The plates were washed with phosphate-buffered saline containing Tween, and serial dilutions of individual serum

samples were incubated for 1.30 h at 37°C. After washing, a 1/700 dilution of rabbit anti-mouse IgG conjugated with peroxidase (Institut Pasteur Production) was added to each well and incubated for 1.30 h at 37°C. The enzymatic activity was determined with the OPD (o-phenylenediamine dihydrochloride) substrate (Sigma) and measured by a microELISA reader (TITERTEK; Flow Laboratories, Inc.). Purified mouse anti-OVA, obtained by affinity chromatography as described elsewhere (18), was used as a standard for the quantification of IgG OVA antibodies in the samples. By this technique, only IgG OVA antibodies could be detected just after the booster immunization in control mice. The data were analyzed by the Scheffe *t* test.

In initial experiments, C3H/HeJ mice were sacrificed 7 days after the booster i.p. immunization, 28 days after OVA or saline ingestion (Fig. 1). The results clearly indicated that OVA feeding markedly suppressed the IgG antibody response in axenic as well as in conventional mice. However, there was a large difference in antibody levels between axenic and conventional control groups. Conventional animals not previously fed with OVA produced over 2 to 3 times the antibody titers to OVA that the axenic animals did ($P < 0.001$).

The maintenance of OVA antibody suppression in tolerized axenic and conventional mice was monitored for a 3-month period after OVA feeding (Fig. 2). After the challenge immunization, performed 60 days after the oral dose of OVA, antibody levels increased significantly in the axenic tolerized group ($P < 0.01$), while a continued suppression of a humoral response was maintained in conventional mice throughout the observation period. It is interesting that the antibody levels observed in tolerized axenic mice after the third challenge injection were not significantly different from those achieved initially in axenic control mice after the second injection (Fig. 2B).

Several authors have reported that oral tolerance to SRBC could not be induced in conventional LPS-unresponsive C3H/HeJ mice (9) and axenic BALB/c mice (23). It was suggested that the gut microflora, especially gram-negative bacteria, plays an important role in the induction of oral tolerance (23). The results presented here show that the presence or absence of digestive microflora does not modify the ability to induce oral tolerance to OVA in LPS-unresponsive C3H/HeJ mice. Thus, the importance of the gut flora for the induction of oral tolerance may depend on the antigen used.

Suppression of the production of anti-OVA IgG in re-
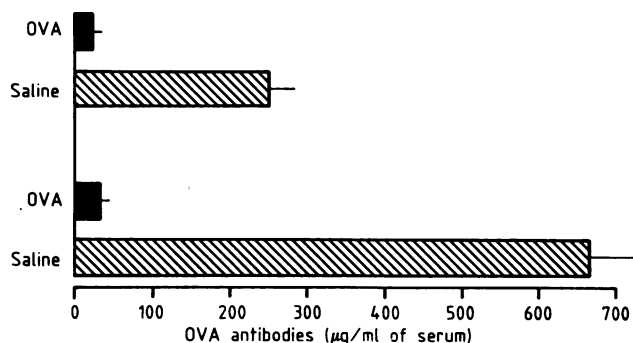
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* Corresponding author.

FIG. 1. Effect of the digestive microflora on the induction of OVA oral tolerance in C3H/HeJ axenic (top) and conventional (bottom) mice. Symbols: ■, tolerized group (fed with 20 mg of OVA given by intragastric intubation); ▨, control group (fed with saline); —, standard error of the mean (10 mice per group). $P < 0.01$ for all saline values. Mice were killed 28 days after feeding and 8 days after the booster i.p. immunization.

sponse to challenge i.p. doses of OVA was present for as long as 90 days after a single 20-mg oral dose of OVA in conventional mice. Our results show that the absence of the digestive microflora affects the maintenance of oral tolerance. Under the same conditions of OVA feeding and systemic immunizations as those used for conventional mice, an IgG antibody response was visualized in axenic mice after a challenge immunization performed 60 days after the OVA feeding. Our data concern only an IgG response, and it would be interesting to know whether the gut flora is also an immunoregulating factor for the suppression of IgE response (5).

The presence of suppressor cells (T_s cells) in animals given antigens orally has been well demonstrated (8, 13, 15, 17). However, Richman (16) reported that 4 weeks after OVA feeding, T_s cells were not demonstrable in the spleens of fed animals, even though animals remained tolerant to the antigen. A dual specific suppressor mechanism for oral tolerance to SRBC (7) and for epitopic suppression (19) has been demonstrated, and an additional mechanism(s) other than T_s cells could be implicated (3, 13, 14, 16). It is possible that a dual mechanism exists for OVA oral tolerance, and the role of digestive microflora could be to affect maintenance
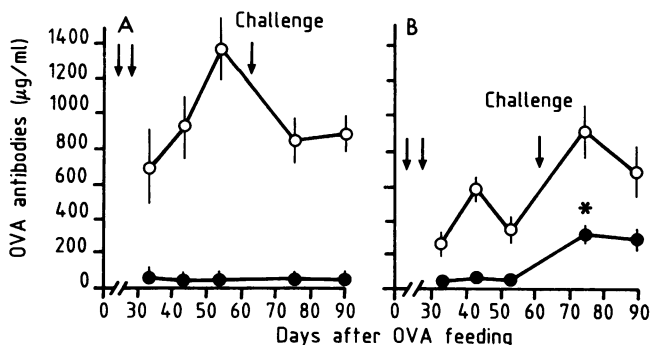

FIG. 2. Effect of the gastrointestinal microflora on the maintenance of oral tolerance to OVA in C3H/HeJ conventional (A) and axenic (B) mice: kinetics of antibody response. Symbols: ●, tolerized group (fed with 20 mg of OVA given by intragastric intubation); ○, control group (fed with saline); ↓, i.p. immunization; *, $P < 0.01$. Values are shown plus or minus the standard error of the mean (10 mice per group).

rather than induction in mice. The ability of the digestive microflora, especially gram-negative bacteria, to stimulate IgA precursor cells in Peyer patches to give rise to IgA-secreting plasma cells in the lamina propria has already been demonstrated (4, 11, 12). However, the effect of the digestive microflora on the precursors of T_s cells in the gut-associated lymphoid tissue which are implicated in oral tolerance (3, 17, 23) is still not clearly defined. The digestive microflora could interfere as an adjuvant or as a specific regulating factor on subsets of T cells of the gut-associated lymphoid tissue.

It is likely that humoral suppression is not initially determined by B cells (14, 16). This concept is supported by the results presented here because, in axenic mice, the level of IgG antibody obtained after challenge when the unresponsiveness state is abrogated is as high as the level in the secondary response. Thus, oral tolerance could not prevent the formation of memory cells. This phenomenon is not observed in conventional mice (20, 22).

The relationships between the digestive microflora and the gut-associated lymphoid tissue are poorly understood (1, 10, 21). However, the microflora is of paramount importance in defining the environment of the gut. Knowledge about the role of the digestive flora in the immune regulation of oral tolerance, as well as the exact mechanisms involved, may help to prevent harmful immunological reactions to food antigens after antibiotic therapy, especially in newborns. The axenic animal will be a valuable model for expanding knowledge in this area.

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