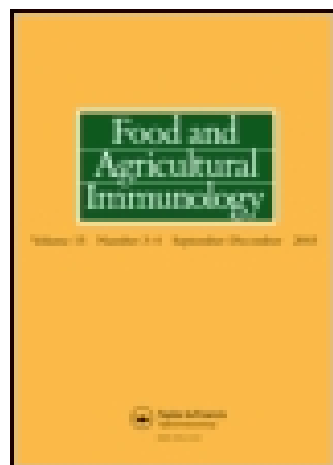




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Allergenicity of Bovine Casein. I: Specific Lymphocyte Proliferation and Histamine Accumulation in the Mastocyte as a Result of Casein Feeding in Mice

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The effects of feeding cow milk casein (100 mg per day) on allergic responses in mice were studied by observing ³H-thymidine incorporation in lymphocytes and histamine release from peritoneal mastocytes. Lymphocytes cultured in the presence of casein showed proliferative activities as early as two weeks feeding up to the eighth week. This activity was not in agreement with either the positive cutaneous test or the antigenic histamine liberation. The total histamine contents in the mastocytes were found to increase from 3.2 ± 1.9 nmol per 10^5 cells for the control group to 7.1 ± 3.2 ; 6.2 ± 1.3 ; 6.7 ± 1.6 after four, six and eight weeks of feeding, respectively. These findings leads us to postulate that there were lymphocyte activations and mastocyte histamine accumulations as the results of intestinal permeability to casein-derived molecules.

Keywords: Antigenic histamine liberation, casein, lymphocyte sensitization, mastocytes, proliferative.

INTRODUCTION

The symptoms of cow's milk allergy (CMA), such as prolonged diarrhoea, vomiting and failure to thrive, were established as early as 1910 (Savilahti & Verkasalo, 1984). In addition, milk is one of the commonest antigens associated with atopic eczema in children (Host, 1987; Ventura & Greco, 1988).

Many attempts have been made to define an immunological mechanism in CMA by measuring antibodies against milk proteins (Harris *et al.*, 1989; Tainio & Savilahti, 1990), lymphocyte transformation (van Sickle *et al.*, 1985; Baudon *et al.*, 1987; Albani

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et al., 1989) and lymphokine production (Weil *et al.*, 1982; Khoshod *et al.*, 1986). These works were performed by using sera of children that were allergic to milk proteins. Some investigations have evaluated the effect of different processing treatments on the allergenicity of milk proteins. In this aspect, oral sensitization and systemic challenge in guinea pig was emphasized (Monneret-Vautrin *et al.*, 1982; Heppel *et al.*, 1984; Pahud *et al.*, 1986). Other researchers have concentrated on the antigenicity of milk proteins using immunochemical methods to determine the antigenic sites (Otani *et al.*, 1986; Otani & Hosono, 1987; Nagaune *et al.*, 1988). Oral presentation of an antigenic material is thought to be a good method of comparing the potential allergenicity of foods for man because different results could be obtained in feeding experiments and by immunochemical assessment (Heppell *et al.*, 1987).

The investigation of food allergenicity should consider the cellular immune response to ingested antigens in a model animal which could represent the natural oral sensitization in human. T-cell stimulation by intestinal derived antigens is a primary step in either reagenic antibody production or cellular-mediated immune responses. Furthermore, the release of histamine from sensitized mastocytes is a result of a specific IgE antibody affinity for the cells and to the corresponding food antigens (Ishizaka & Ishizaka, 1984; Vercelli & Geha, 1989). This interaction serves as a principal base of the hypersensitivity tests, such as systemic and passive cutaneous anaphylaxis (Knicker, 1989). The present study was undertaken to investigate the possible allergenic response to bovine casein by observing the initial T-cell blastogenic activity and IgE-mediated antigen induced histamine liberation of mastocytes. Casein was chosen because it is major milk protein and because hydrolysates are often used in infant diets.

MATERIAL AND METHODS

Mice

Swiss albino mice, three months of age, were purchased from Charles River Breeding Station, Paris. The mice, weighing 18–22 g, were immediately kept on a standard pelleted diet which contained no casein or other milk proteins (CRF Alimentation, Paris) for one month prior to and throughout the feeding experiments.

Casein Preparation

Casein was isolated from fresh cow's milk, supplied by the university farm, using the isoelectric precipitation method. The milk was centrifuged at $500 \times g$ for 30 min, kept at 4°C for 2 h and filtered over cotton wool to separate the cream. The protein was precipitated with 2 M-HCl at pH 4-6 and the precipitate washed with distilled water followed by redissolving with the original amount of distilled water adjusted to pH 7 using 2 M-NaOH. The precipitation and redissolving process was repeated twice before the final sodium caseinate solution was lyophilized.

Feeding Experiments

Four groups of eight to ten mice were tube fed with 1 ml of 10% casein in distilled water each morning of the week days for 2, 4, 6 and 8 weeks (group 2, 3, 4, 5 respectively). Another group of mice was kept to serve as a control group (group 1), and these animals did not receive casein. At the end of the feeding treatments the animals were sacrificed by vertebral dislocation.

Intraperitoneal Immunization

Another group of six mice received intraperitoneal injection of 1 ml 5% casein in 0.15 M-NaCl solution. The injections were carried out twice a week for four weeks. The mice were sacrificed a week after the last injection.

Skin Reactions

Casein (100 μg in 0.1 ml, 0.15 M-NaCl solution) was injected intradermally into the shaved back of the mice. Skin reactions were assessed one and 24 h later by observing skin erythema.

Preparation of Cell Suspensions

After being sacrificed, the animals were wiped with 90° alcohol and kept under a sterile laminar flow hood. Mastocyte suspension was prepared by injection of 5 ml sterile Tyrode's solution (mm: NaCl 137, NaHCO_3 12, glucose 5.6, KCl 2.7, NaH_2PO_4 0.4, MgCl_2 1 and CaCl_2 1.8) into the peritoneal cavity. After light massaging for 2 min, the liquid was aspirated and centrifuged at $150 \times g$ for 10 min (Siraganian & Hazard, 1979). The pellet was then resuspended in 2 ml of Tyrode-BSA-HEPES solution containing 0.1% bovine serum albumin (BSA) and 10 mM-HEPES buffer (Befus *et al.*, 1982). The cells were counted after staining with toluidine (0.1% in 50% v/v ethanol, pH 3) as colourant, and adjusted to 3×10^5 cells per ml by pooling peritoneal liquid from several mice.

Following aspiration of the peritoneal liquid, mouse skin was incised to remove the spleen, which was immediately washed and gently crushed in 5 ml of RPMI-1640 solution (Boehringer, Mannheim) in a potter tube. After separating tissue debris with three layers of gauze, the suspension was centrifuged at $150 \times g$ for 10 min, and the cells were resuspended in 10 ml RPMI 1640 supplemented with 10% heat inactivated foetal calf serum (Boehringer) 100 μg ml^{-1} streptomycin and 100 U ml^{-1} penicillin (Specia, Paris). The cells were counted by using trypan blue, which showed 99% viable cells, and adjusted to a suspension of 5×10^6 mononuclear cells per ml.

Histamine Release

Histamine release was assayed by the method of Siraganian & Hazard (1979). The mastocyte suspension (10^5 cells, 300 μl) was put into tubes and 200 μl of Tyrode-BSA-HEPES buffer containing added casein which gave a final antigen concentration of 10, 20, 30, 40 or 100 μg ml^{-1} . Two other tubes were set up with the addition of buffer alone. The first tube was to serve as control (unstimulated release) and the second was used for the measurement of total histamine content. The tube containing antigen and the control tube were incubated in water bath at 37°C for 10 min. The tube for total histamine was placed in boiling water for 3 min. The reactions were stopped by cooling immediately in ice bath followed by centrifugation at $100 \times g$ for the control tube and the tube containing the antigen. The tube for total histamine content were centrifuged at $300 \times g$ for 10 min. Histamine concentration was determined from supernatant by radioimmunoassay using a commercial kit provided by Immunotech, Marseille.

Lymphocyte Culture

Cell cultures were set up in quadruplicate on flat bottomed microtitre plates (Falcon, Grenoble). Into each well were placed 100 μl of lymphocyte cell suspension (5×10^5 cells) and 100 μl of casein solution in RPMI 1640 to give a final concentration of 50 or 100 μg ml^{-1} . In the preliminary experiments, we cultured sensitized lymphocytes (from mice fed with casein for two weeks) at concentrations of casein of 50, 100, 150,

200 and 250 $\mu\text{g ml}^{-1}$. The first two concentrations were found to be optimal. Control (containing 100 μl of RPMI 1640 alone) and phytohaemagglutinin (PHA, Difco (Paris), 100 μl of 1:100 dilution) cultures were performed simultaneously. The cells were incubated at 37°C in 5% CO_2 for four days. For the last 18 h, 0.1 μCi of ^3H -thymidine (specific activity 1.08 mCi mmol^{-1} , Amersham, les Ullis) were added to the cultures. Cells were collected onto glass fibre filters (Whatman GF/C) and washed 10 times with 0.5 M-NaCl solution by using a semi-automatic harvester (Titertek Skatron). The discs containing the cells were air dried and dissolved in 0.5 ml soluene 350 (Packard) for 1 h. The radioactivity was counted in a Beckman scintillation counter by using 4.5 ml scintillation solution (Insta-Fluor, Packard, Rungis). Results were expressed as stimulation index (SI), where SI is the mean count per minute (cpm) of stimulated culture divided by the mean cpm of control culture.

Statistical Analysis

Differences due to feeding treatments and types of stimulants were determined with a two-way analysis of variance and Duncan's multiple-range test.

RESULTS

Dose Response Curve of Histamine Release

Incubation of mastocytes from mice fed with casein for two weeks with the antigen at concentrations of 0 to 100 $\mu\text{g ml}^{-1}$ did not modify significantly the increase of histamine released from the cells (Figure 1). However, we decided to continue the experiments with the cells from prolonged casein-fed mice using casein at 20 $\mu\text{g ml}^{-1}$.

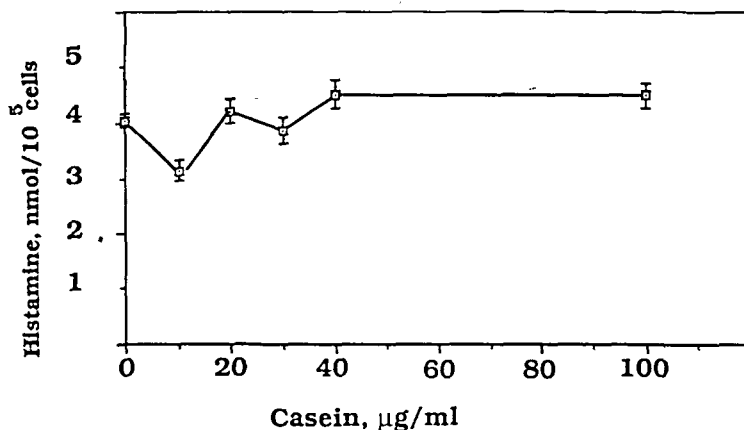


FIG. 1. Histamine liberation from mast cells of mice fed with casein for two weeks and incubated in the presence of different antigen concentrations. Each value represents the mean of eight replications with mastocytes pooled from four mice.

Histamine Release

When the mastocytes of mice fed with casein for different periods of time were incubated with 20 $\mu\text{g ml}^{-1}$ of casein, the quantity of histamine released was not statistically different from histamine released spontaneously ($p > 0.05$, Figure 2). This pattern could be observed in almost every mouse in each group. However, when the

intracellular histamine concentration was high, the histamine release either spontaneously or in presence of casein was also high. It could be postulated that the intracellular histamine was a factor of allergy severity.

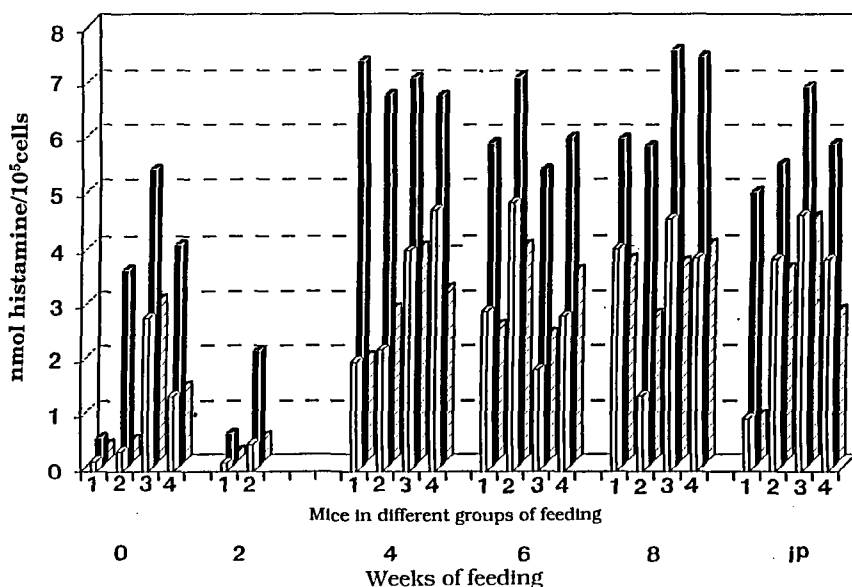


FIG. 2. The quantity of histamine released and total histamine content of mastocytes from mice fed with casein for different periods of time. Each value is the mean of eight assays performed from four mice. (□) histamine released from cells incubated in control medium; (■) total histamine content of cells incubated in control medium; (▨) histamine released from cells incubated in the presence of casein.

The amounts of the total histamine in the cells of mice fed with casein were found to increase significantly ($p < 0.01$) as the feeding period was extended. These increments were from 3.2 ± 1.9 nmol per 10^5 cells in the control group to 7.1 ± 3.2 ; 6.2 ± 1.3 ; 6.7 ± 1.6 after four, six and eight weeks of feeding respectively. Figure 2 also demonstrates that repeated intraperitoneal injections in mice lead to histamine accumulation in the peritoneal mastocytes as well. This accumulation was statistically significant as compared to that of the control group ($p < 0.01$).

Skin Reactions

The results of skin observation 1 h and 24 h after challenging with $100 \mu\text{g}$ of casein on the backs of mice did not show any signs of skin erythema. These results were in accordance with the negative IgE-mediated histamine release test from mice peritoneal mastocytes.

Effect of Casein on Lymphoblastic Response

The lymphoblastic responses expressed as stimulation index (SI) to casein of all groups of mice is presented in Figure 3. The cells from all groups, including the control, responded actively to casein stimulation. The geometric means of ^3H -thymidine incorporation by lymphocytes cultured in medium alone were 4372 ± 619 , 1216 ± 224 , 2341 ± 231 , 1307 ± 291 and 2841 ± 334 cpm, respectively, for mice fed with casein for zero (control), two, four, six and eight weeks. The *in vitro* responses to the two casein

concentrations (50 and 100 $\mu\text{g ml}^{-1}$) were statistically the same in all groups. In all cases, PHA stimulation was always higher than that of casein ($p < 0.01$). However, neither treatment statistically influenced the response to this mitogen. Figure 3 also shows that lymphoblastic response to casein by mice receiving the antigen intraperitoneally was much lower than that of those administered orally with casein.

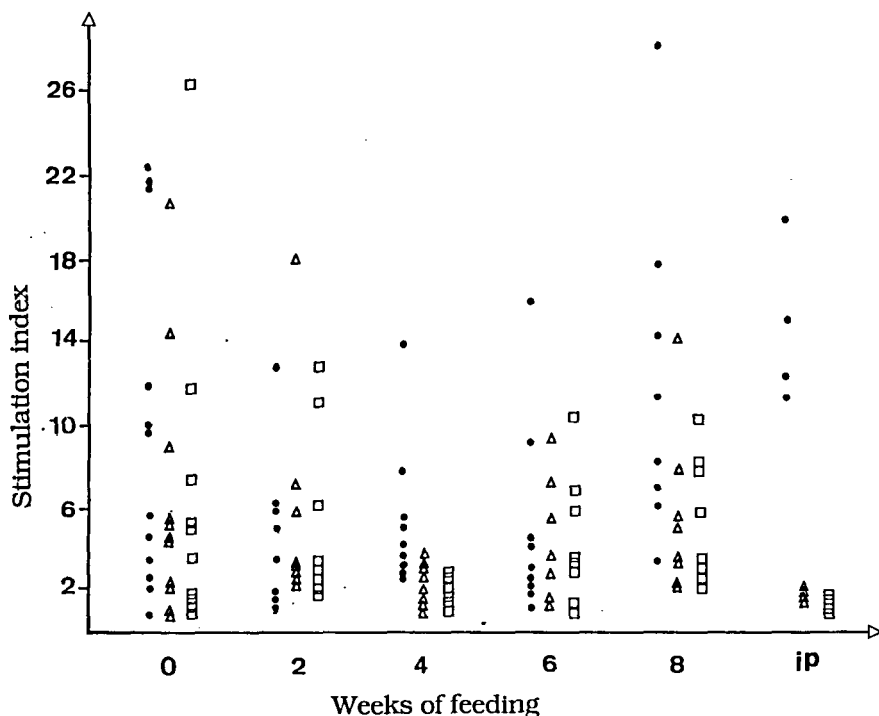


FIG. 3. Mitogenic activity of casein in lymphocytes from mice fed with casein for different periods of time or immunized intraperitoneally (IP). Phytohemagglutinin (PHA) was used as a positive control. The cells were cultured in the presence of: (●) PHA; (Δ) casein 50 μg ; ($\square$) casein 100 μg . Each value is the mean of four assays performed from one mouse. The coefficients of variation ranged from 7% to 20%.

DISCUSSION

In order to investigate the presence of IgE-mediated reactions in mice after feeding with casein, we used peritoneal mastocytes instead of mucosal mastocytes, although the latter might be more representative in a feeding study because they could be in more direct contact with food antigens in the digestive tract. However, peritoneal mastocytes could be isolated more easily with a high degree of purity. Befus *et al.* (1982) have shown that both sensitized peritoneal and mucosal mastocytes possess an equal capacity to release histamine in the presence of the specific allergen. The histamine release test, using mast cells, represents a more precise approach to an *in vivo* immunological degranulation and it permits the detection of specific IgE fixed on the cell membrane (Weck, 1988). This *in vitro* allergic test and skin reaction tests are preferable to either RAST or skin testing alone in diagnosis of allergic diseases (Knicker, 1989).

The results of the histamine release test in this study which showed no specific release is in accordance with the negative results of the skin test. This suggests an absence of specific IgE anti-casein antibody production albeit after a prolonged consumption of casein (100 mg per day). As casein is a known histidine rich protein (2.7 g%) (Poiffait & Adrian, 1986), so continuous consumption of casein in mice could induce an increase in histidine absorption by the intestine which could then be transformed into histamine and accumulated in the histidine decarboxylase-rich mast cells (West, 1986). The casein which was introduced intraperitoneally might experience some degree of hydrolysis leading to histidine and/or histamine production. The absorption of radiolabelled histamine into mouse mast cells had been shown (West, 1986). The incidence of high plasma histamine levels in allergic individuals after the ingestion of allergens had been reported (Sampson & Jolie, 1984). Since mastocytes can be induced to release their biogenic compounds by other stimulants, i.e. certain T-cell derived factors, beside specific IgE (West, 1986), the mechanism of this histamine accumulation in the mast cells and its significance in allergic manifestations needs to be verified.

Lymphocyte proliferation tests (LPT) have been used to determine cellular sensitization to cow's milk protein and are a measure of a role of T-cells in the pathogenesis of the reactions (Tainio & Savilahti, 1990; Albani *et al.*, 1989; Baudon *et al.*, 1987; Weck, 1988). The specificity of the proliferative response in the presence of the sensitizing antigen has been recently established. Phillips *et al.* (1987) had demonstrated the restriction of antigen E-specific T-cells to ragweed-allergic donors by culturing the cells for a longer period of time and providing a second *in vitro* boost with the antigen. The stimulation of T-cells is also a primary step in the production of IgE, which is a reaginic antibody. The results presented in this paper showed no correlation between lymphocyte proliferation, and specific IgE-mediated histamine release from mastocytes might indicate that the T-cell activation as a result of casein consumption was a manifestation of an IgE-independent cellular response. Similar results had also been reported by others. Studies of patients with symptoms of cow's milk allergy do not show a correlation between lymphocyte proliferation and the concentration of serum IgE (Albani *et al.*, 1989; Tainio & Savilahti, 1990). Tainio & Savilahti (1990) suggested that early exposure to cow's milk might lead to suppression of IgE responses but at the same time provoke cellular reactions which might later induce allergic symptoms. Most of the results for LPT performed with CMA children seem to indicate that the positivity of the test correlates closely to CMA with perturbation at the intestinal level. Van Sickle (1985) reported the evidence of lymphocyte sensitization by casein in children with enterocolitis. More recently, Baudon *et al.* (1987) found an elevated LPT response in patients with gastrointestinal symptoms. A working group of the Italian Society of Pediatrics (Ventura & Greco, 1988) had indicated that specific IgE were usually absent in chronic enteropathic disorders. They divided the 303 CMA children into two groups; an atopic group with high IgE level and a gastrointestinal group with lower prevalence of specific IgE. These data suggest a relationship between lymphocyte activation and an IgE-independent cellular mechanism at the intestinal level. This hypothesis has encouraged us to probe into the intestinal absorption of casein-derived antigens (manuscript in preparation).

Several arguments could be presented to explain the positive lymphoblastic response of the control mice. In this experiment, we put the animals on a casein-free diet once they were two months old. During the first month of life, before their arrival in the laboratory, the mice were kept on a standard animal feed which contained milk proteins including casein. Sensitization of laboratory animals to certain nutrient components in the animal feed has been reported (Coomb *et al.*, 1983). The fact that casein had been consumed by the mice from a very young age, when the immaturity of intestinal mucosa permits the passage of large food protein molecules (Walker, 1987), followed by a one-month casein-free period, might explain the lymphocyte sensitiza-

tion of the control group mice. It was also reported that feeding albumin in small quantities induced a specific response in newborn mice (Strobel & Ferguson, 1984). Furthermore, the passage of maternal dietary proteins into the breast milk in humans (Kilshaw & Cant, 1984) and the effect of breast-feeding on the development of food protein antibodies in neonate rats (Okamoto *et al.*, 1989) had been established. It was possible that our mice had been sensitized by casein from a very young age when the mucosal permeability allows food protein penetration. This sensitization could have occurred through continuous consumption of this protein in small quantities from the mother's milk and through the standard animal diet during weaning. The sensitization was maintained by the feeding treatment but seemed to be eliminated by intraperitoneal injection after a period of resting. Results from our further investigation on lymphocyte response to casein in mice which have never consumed any milk protein for a generation support this hypothesis (manuscript in preparation).

The fact that murine lymphocytes fed with casein showed a positive [^3H]thymidine incorporation induced by the same antigen indicates that *in vivo* cell activation is caused by any casein or its fragments which were able to penetrate the intestinal mucosa. However, intraperitoneal injection of casein did not provoke lymphocyte activation. Depending on the route of antigen introduction, the stimulation of the immunocompetent cells might occur in different lymphoid organs (Lake, 1983). There is also a possibility, as previously reported (Bruce & Ferguson, 1986a), that ingested antigen may be filtered by the intestinal mucosa which can determine the immunological characteristic of a protein fragment or molecule which managed to achieve a lymphoid organ. This intestinal processing was not experienced by the antigen introduced intraperitoneally. It was also observed previously that large amounts of antigen injected parenterally into newborns tended to lead to tolerant responses (Strobel & Ferguson, 1984). It would be interesting to consider this negative lymphocyte response as an indication of tolerance against casein, as it was suggested from experiments in mice that in clinical food-sensitive enteropathy there was an intestinal immune response gene-controlled defect in tolerance to dietary protein (Mowat *et al.*, 1987). The role of the intestinal antigen processes during absorption and antigen presentation at the local lymphoid organ might be crucial in determining the antigen immunogenicity. It was reported earlier that intestinal processing influenced the immunogenicity and molecular size of absorbed, circulating ovalbumin in mice (Bruce & Ferguson, 1986b). The exact mechanism of the immune induction by digestive and intraperitoneal paths of antigen introduction would be important to elaborate in order to understand better the mechanism of food protein tolerance and sensitization.

CONCLUSION

Casein consumption in mice did not provoke an IgE-mediated reaction such as specific histamine release from mastocytes or an erythematous response in cutaneous tests. It resulted in histamine accumulation in the mastocytes and lymphocyte sensitization which was expressed in a lymphocyte proliferation test. Feeding of casein to already sensitized mice could re-establish the sensitization, however, intraperitoneal injection seemed to desensitize animals. These results suggest the intestinal passages of casein-derived antigens which activated the lymphocytes and the important role this intestinal processes has in determining whether the immune response is tolerant or priming in nature.

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