

Immunodeficiency and other clinical immunology

Induction of systemic immunologic tolerance to β -lactoglobulin by oral administration of a whey protein hydrolysate

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Background: Oral administration of an antigen has been shown to suppress the specific immune response to this antigen. This approach, called oral tolerance, has been demonstrated with intact proteins in animal models for prevention of allergy and autoimmune diseases.

Objective: The purpose of this study was to determine whether oral tolerance can be induced with protein peptides. Partially hydrolyzed and extensively hydrolyzed cow's milk formulas were compared for their capacity to induce tolerance to cow's milk proteins.

Methods: Five-week-old Sprague-Dawley rats were fed cow's milk formulas ad libitum from day 1 to day 19. All animals were immunized with β -lactoglobulin and ovalbumin on day 5 and bled on day 19. Sera were analyzed for specific IgE and IgG antibodies by ELISA and for functional IgE response by in vitro mast cell mediator (tritiated serotonin) release. In vivo modulation of intestinal mast cells was analyzed by the specific release of the rat mast cell protease II, and T-cell response was determined by tritiated thymidine incorporation into lymph node lymphocytes.

Results: Oral administration of a partially hydrolyzed cow's milk formula suppresses specific serum IgE and IgG anti- β -lactoglobulin antibodies, as well as mediator release from rat mast cells and T-lymphocyte response. This suppression was shown to be antigen-specific and dose-dependent. An extensively hydrolyzed formula was unable to achieve the induction of such an oral tolerance.

Conclusion: These results support the view that partially hydrolyzed proteins are able to induce specific oral tolerance, whereas extensively hydrolyzed proteins are not. (*J Allergy Clin Immunol* 1997;100:266-73.)

Key words: Oral tolerance, β -lactoglobulin, whey protein, hydrolysate, IgE, mast cells

Oral administration of protein antigens induces specific immunologic hyporesponsiveness (tolerance) to these antigens. Induction of oral tolerance with intact proteins has been well documented with a number of antigens in several animal models. In addition to the first guinea pig experiments done by Wells and Osborne,¹

Abbreviations used

β LG:	β -Lactoglobulin
HEPES:	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
PHG:	Phosphate-HEPES-gelatin
RMCP II:	Rat mast cell protease II buffer
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis

specific oral tolerance to bovine serum albumin has been shown in a Black Norwegian rat model.² The same phenomenon has also been described in a mouse model, in particular by Hanson et al.³ More recently, oral tolerance has been established in human beings. When human volunteers were fed keyhole limpet hemocyanin, oral tolerance was observed at the T-cell compartment, assessed by reduction of antigen-specific T-cell proliferation and markedly diminished delayed-type skin test reactivity in the keyhole limpet hemocyanin-fed group.⁴

Immune regulation by the induction of oral tolerance to food antigens is thought to prevent food allergy.⁵ It has been shown that induction of oral tolerance is dependent on the age of the host,⁶ the dose of antigen administered,⁶ and the nature of the antigen. Although oral tolerance to dietary proteins has been extensively investigated with intact antigens, few studies with antigen fragments⁷ or digests⁸ have been done.

In this study we attempted to determine whether enzymatic hydrolysates of cow's milk (whey) proteins could induce oral tolerance to these proteins, when administered preventively ad libitum in rats. We selected β -lactoglobulin (β LG) as a representative protein for immune modulation studies because it is one of the most potent allergens of cow's milk⁹ and because it quantitatively represents the main whey protein fraction.

Our results show that a partially hydrolyzed formula can induce specific oral tolerance, whereas an extensively hydrolyzed formula cannot.

METHODS

Reagents

Bovine β LG (No. L-0130), o-phenylenediamine dihydrochloride, fish gelatin, N-2-hydroxyethylpiperazine-N-2-ethanesul-

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fonic acid (HEPES), and peroxidase-labeled donkey anti-sheep IgG were from Sigma Chemical Company (St. Louis, Mo.). Sheep anti-rat IgE was purchased from ICN Immuno Biologicals (Lisle, Ill.). Tritiated serotonin (5-hydroxy-tryptamine creatinine sulfate) and tritiated thymidine were obtained from Amersham International (Buckinghamshire, U.K.). Dulbecco's modified Eagle's medium, fetal calf serum, antibiotic mixture, and L-glutamin were from Fakola-Seromed (Basel, Switzerland). Aluminium hydroxide gel containing 3% Al(OH)₃ was from Superfos, Denmark.

Milk formulas

NAN (Nestlé) is a standard cow's milk formula based on intact casein and whey proteins (40:60 weight ratio). BEBA-HA (Nestlé) is an enzymatically (trypsin) hydrolyzed whey formula (18% hydrolysis). ALFARE (Nestlé) is based on a pancreatic hydrolysate (28% hydrolysis) of isolated whey proteins.

Animals

Four-week-old male Sprague-Dawley rats were purchased from Iffa-Credo (Arbresle, France). They were bred and raised on a milk-free diet. At the onset of the experiment, the rats were 5 weeks old.

Experimental design

Modulation of antibody or T-cell response. The standard experimental protocol was as follows. From day 1 to day 19, groups of eight rats were given different experimental liquid milk formulas or water (control) ad libitum in their drinking bottles and a solid "milk-free" pellet diet. The groups were given the following products: group A, NAN; group B, BEBA-HA; group C, ALFARE; and group D, water. All formulas were administered at a concentration of 120 gm/L, except in the experiment testing the dose dependency in which formulas with concentrations of 0.12, 1.2, 12, or 120 gm/L were orally administered. All animals were immunized on day 5 by subcutaneous administration of 0.1 mg β LG and 0.1 mg ovalbumin (as an unrelated antigen for testing the specificity of the immune response modulation) in 0.05 ml of saline solution mixed with 0.2 ml 3% Al(OH)₃. Fourteen days later, at the peak of the IgE primary response in rats, all animals were killed. Blood was drawn, and sera were kept frozen at -80° C until specific IgE and IgG anti- β LG or anti-ovalbumin antibodies were determined by ELISA analysis. Inguinal lymph nodes were removed, and T lymphocytes were purified for stimulation assays.

Intestinal mast cell modulation. The following changes in the above-mentioned protocol were made. Rats were fed the experimental cow's milk formulas from day 1 to day 14 only, and on day 19 of the experiment, were given an oral challenge with a whey protein concentrate (1 gm/2 ml) 2 hours before final bleedings. Rat mast cell protease II (RMCPII) levels were determined with a commercial ELISA kit (Moredun Animal Health Ltd., Edinburgh, Scotland), based on the sandwich test principle in which the plate coating is made with a monoclonal anti-RMCPII antibody, followed by the addition of test serum and a second sheep anti-RMCPII antibody coupled to horseradish peroxidase.

ELISA determinations of specific rat IgE and IgG antibodies

Analyses of specific rat IgE and IgG antibodies were done as previously described.¹⁰ In brief, microtitration plates were coated with the antigen (β LG or ovalbumin) for 24 hours at 4° C. The unoccupied sites on the plate were blocked with fish

gelatin. Four washing steps with phosphate-buffered saline-Tween-20 were performed before each successive reagent addition. After addition of test sera dilutions and incubation for 2 hours, a sheep anti-rat IgE (for IgE determination) or goat anti-rat IgG (for IgG determination) antiserum was added. After 1 hour at room temperature, a peroxidase-labeled second antibody was added for 1 hour, followed by the substrate (o-phenylene-diamine). The optical density of a 1:5 diluted pool of normal rat sera at 492 nm was considered as nonspecific background. Concentrations of specific antibodies were expressed as maximum dilutions of test sera above this value. The specificity of the anti-rat IgE was tested with the rat IgE myeloma IR-2¹⁰ and was not found to cross-react with polyclonal rat IgG. Species specificity of the peroxidase-labeled conjugates was also checked.

In vitro tritiated serotonin release assay

The efficacy of IgE suppression was tested by the passive sensitizing capacity of sera from orally tolerized or control animals on peritoneal mast cells. The peritoneal mast cells from normal rats were sensitized with sera from the different experimental groups, washed, labeled with tritiated serotonin, washed again, and triggered for mediator release by incubation with β LG dilutions according to a published method.¹⁰ In brief, peritoneal lavages were obtained with Dulbecco's modified Eagle's medium plus 10% fetal calf serum. Rat mast cells present in the lavages were washed and kept in this medium overnight at 4° C. After two washes in phosphate-HEPES-gelatin (PHG) buffer, pH 7.0, cells were resuspended at 500,000/ml. Tritiated serotonin (10 μ Ci/ml) was added, followed by 1 ml of rat serum from an experimental group. This mixture was incubated at 37° C for 2 hours and then washed three times and resuspended in 2 ml of PHG buffer. Triggering for mediator release was set up in microtiter plates by mixing 0.05 ml of triggering agent (β LG) with 0.1 ml of sensitized mast cells. The mixture was incubated for 60 minutes at 37° C and centrifuged. An aliquot (0.05 ml) of the supernatant was counted for radioactivity (beta counter).

Lymphocyte stimulation

Lymphocyte suspensions were teased from inguinal lymph nodes with a 10 ml tissue grinder. Cell suspensions were washed three times with Dulbecco's modified Eagle's medium containing 5% fetal calf serum before resuspension in this culture medium. Lymphocytes (0.1 ml at 500,000 cells/well) were incubated with dilutions of β LG (0.05 ml/well) in flat-bottom microculture plates (Costar). Triplicate cultures were incubated at 37° C in a humidified 5% CO₂ atmosphere for 72 hours. The cultures were pulsed with 1 mCi/well methyl-tritiated thymidine (2 Ci/mmol; Amersham, U.K.) 6 hours before harvesting with an automated cell harvester (PHD Cell Harvester; Cambridge Technology, Watertown, Md.), and radioactivity of samples was determined by liquid scintillation counting.

Statistics

The data were tested for significance by Fisher's exact test.

RESULTS

Characterization of cow's milk formulas

When NAN was analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a precast 4% to 12% concentration gel (Novex), bands of various molecular weights, corresponding to intact cow's milk proteins, appeared (Fig. 1). In comparison, SDS-

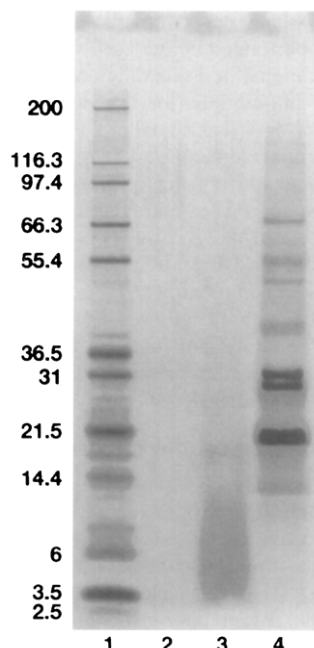


FIG. 1. Characterization of cow's milk formulas by SDS-PAGE. Lane 1, Molecular weight markers (at left of lane 1 molecular weights are indicated in kilodaltons); lane 2, ALFARE; lane 3, BEBA-HA; lane 4, NAN. All formulas were analyzed at a concentration of 0.8 mg of protein equivalent per 10 ml of solution applied.

PAGE of BEBA-HA showed mainly low molecular weight bands, and no bands were seen with ALFARE.

We also measured the allergenicity of these formulas with the help of the tritiated serotonin release assay. The level of β LG-specific IgE-mediated allergenicity of NAN, BEBA-HA, and ALFARE was determined by the capacity of these formulas to trigger tritiated serotonin release from sensitized mast cells. To test this, peritoneal mast cells from normal rats were sensitized passively in vitro with a pool of rat sera containing specific IgE anti- β LG and labeled with tritiated serotonin. Fig. 2 shows that when such mast cells are then triggered with the three formulas, different dose-dependent tritiated serotonin release curves are obtained. One can see that 1000 times more BEBA-HA than NAN, calculated on a protein equivalent basis, is needed for triggering release of the same amounts of tritiated serotonin. ALFARE has a very low triggering activity at a protein equivalent of 10 mg/ml. Calculated values of residual β LG-specific allergenicities in NAN, BEBA-HA, and ALFARE are 100, 0.1, and 0.0002 mg β LG/gm protein equivalent, respectively.

Specific antibody suppression

The three cow's milk formulas were compared for their capacity to induce antigen-specific oral tolerance. Although, as shown above, BEBA-HA is 1000

times less allergenic than NAN, we have now found (Fig. 3) that NAN and BEBA-HA have similar oral tolerizing capacities. This appears particularly at the IgE anti- β LG antibody level: NAN and BEBA-HA are able to suppress specific IgE levels over 100-fold when administered preventively ad libitum by the oral route. In contrast, ALFARE, the extensively hydrolyzed formula, has no tolerizing activity: no IgE suppression against β LG was observed when rats were fed this formula, if compared with the control group (given only water). The specific IgE anti- β LG response of animals fed NAN or BEBA-HA was significantly different ($p < 0.00001$) from the responses of those fed ALFARE or given only water. The effect of formula feeding on the IgG anti- β LG response is weaker, but the same comparative profile as that obtained for IgE modulation is observed. Differences of IgG anti- β LG responses between groups fed NAN or BEBA-HA and groups fed ALFARE or given water only were again highly significant ($p < 0.00001$). Antibody responses to ovalbumin remained unchanged in all groups.

Importance of formula concentration

In a second set of experiments, we analyzed the dose dependency for induction of oral tolerance. We found (Fig. 4) that the same doses of either the standard formula (NAN) or the partially hydrolyzed formula (BEBA-HA) are necessary to obtain similar oral tolerance. The oral ad libitum administration of these formulas at 120 gm/L induces the highest antibody suppression with an IgE anti- β LG antibody titer reduction of over 200-fold when compared with controls (water group = 0 gm/L). This difference is highly significant ($p < 0.00001$). Significant differences were observed between 120 gm/L and 12 gm/L ($p = 0.004$), 12 gm/L and 1.2 gm/L ($p = 0.0004$), 1.2 gm/L and water ($p = 0.025$). Fig. 4 also shows that at all concentrations tested, antibodies to an unrelated antigen (ovalbumin) were not suppressed by feeding of milk formulas, demonstrating that this induction of oral tolerance is allergen-specific.

Suppression of in vitro mast cell secretion

Normal peritoneal mast cells were labeled with tritiated serotonin and passively sensitized with sera of either tolerized or control rats. Sera of rats in which tolerance was induced in vivo with NAN or BEBA-HA failed to sensitize normal rat mast cells for tritiated serotonin release when triggered with β LG (Fig. 5), indicating that low levels of specific IgE anti- β LG antibodies of sera from tolerized animals are quantitatively and/or qualitatively unable to sensitize passively mast cells for β LG triggering. On the contrary, sera from rats pre-fed with ALFARE or control rats were able to sensitize mast cells, which led to tritiated serotonin release. This is because these animals were not tolerized and therefore had high specific serum IgE levels after parenteral sensitization.

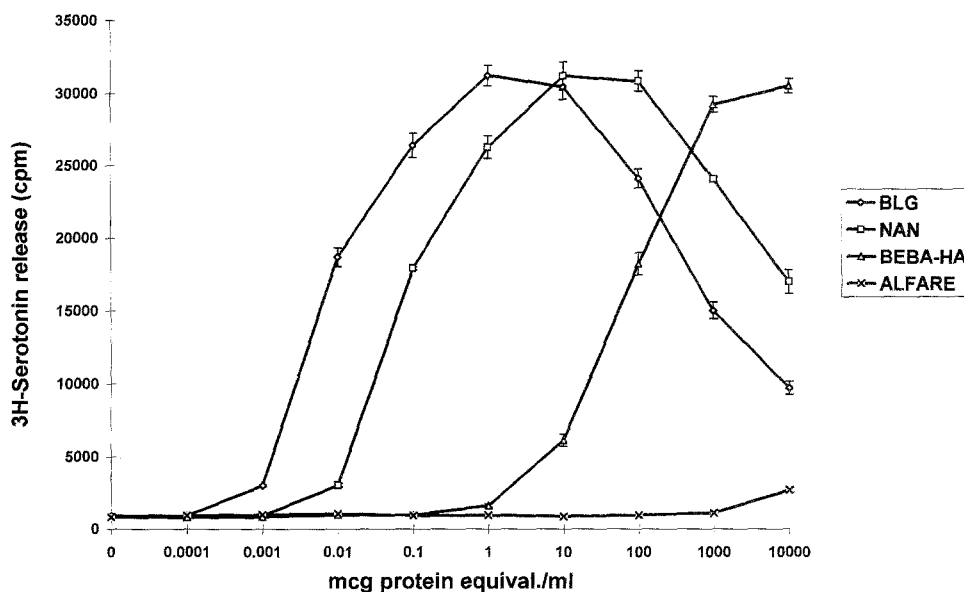


FIG. 2. Residual βLG-specific allergenicity in cow's milk formulas. This was tested by tritiated serotonin release from rat mast cells sensitized passively with rat IgE anti-βLG antibodies. Dose-dependent release curves obtained by triggering sensitized mast cells with BLG or the three formulas are shown.

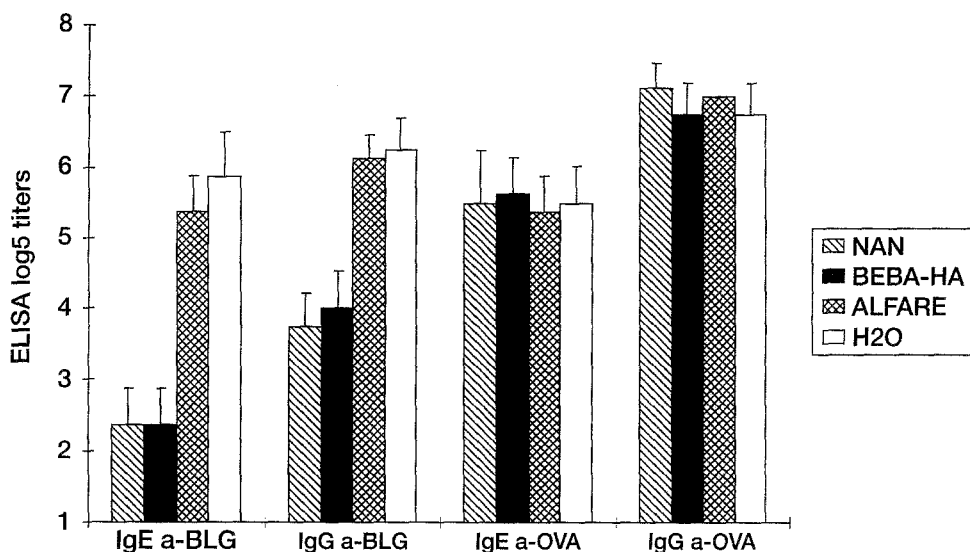


FIG. 3. Antibody suppression by oral tolerance with cow's milk formulas. ELISA (log5) titers of IgE and IgG anti-βLG or anti-ovalbumin antibodies in sera from rats orally tolerized with NAN, BEBA-HA, ALFARE, or water.

In vivo suppression of intestinal mast cell response

We showed (Fig. 6) that the specific secretion of the intestinal mucosal mast cell protease (RMCP II) of sensitized rats could be suppressed by pre-feeding with intact (NAN) or partially hydrolyzed cow's milk proteins (BEBA-HA). This was observed in three different experiments when compared with the control group (given water only). The specific levels of RMCP II released

from intestinal mast cells of rats tolerized with NAN or BEBA-HA were significantly different ($p < 0.00001$) from levels released from mast cells of intolerized rats (control group given only water).

Suppression of lymphocyte proliferation

To test the effect of oral tolerance induction with cow's milk formulas on cellular immunity, a T-cell proliferation assay was done with cultured lymph node cells from rats

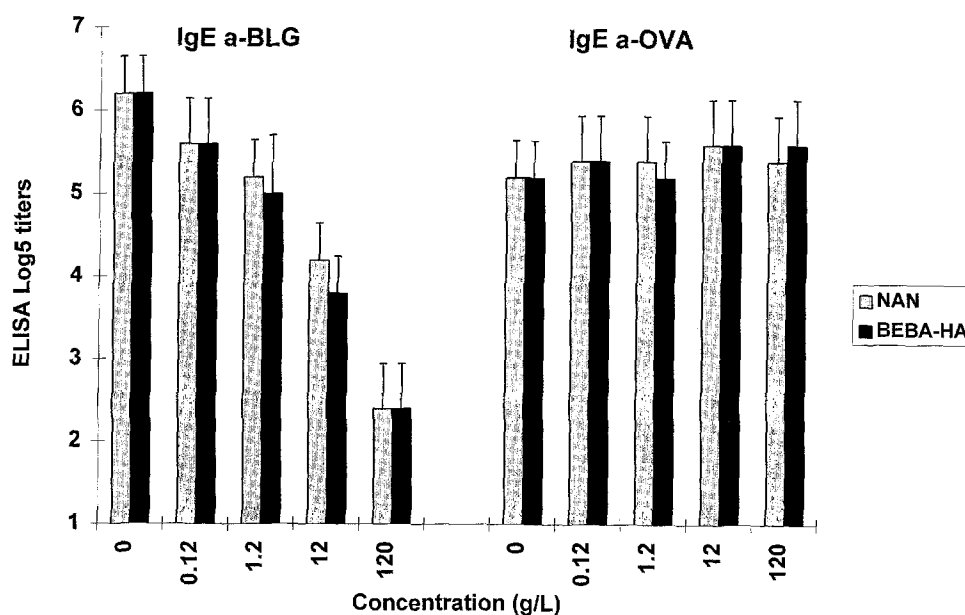


FIG. 4. Dose dependency of antibody suppression. ELISA (log5) titers of IgE anti- β LG or anti-ovalbumin antibodies in sera from rats orally tolerized with increasing doses (0 to 120 gm/L) of NAN or BEBA-HA. BLG, β -Lactoglobulin; OVA, ovalbumin.

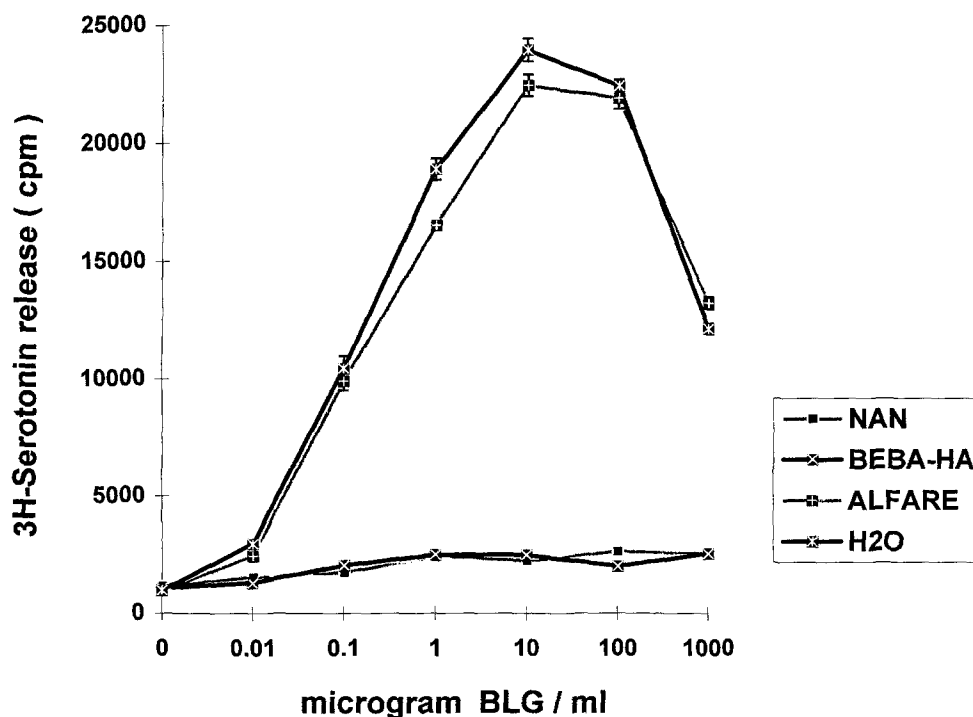


FIG. 5. In vitro suppression of mast cell response. Tritiated serotonin release from mast cells passively sensitized with sera of rats orally tolerized with NAN, BEBA-HA, ALFARE, or water. Triggering was done with β LG (0.01 to 1000 μ g/ml).

pre-fed with 120 gm/L concentrations of the three formulas or from control rats according to the experimental design. One can see on Fig. 7 that lymph node lymphocytes from rats tolerized with NAN or BEBA-HA cannot be stimu-

lated by β LG for tritiated thymidine incorporation. On the contrary, lymphocytes from rats fed with ALFARE or lymphocytes from control rats, cultivated in the presence of β LG, are stimulated according to a dose-response curve.

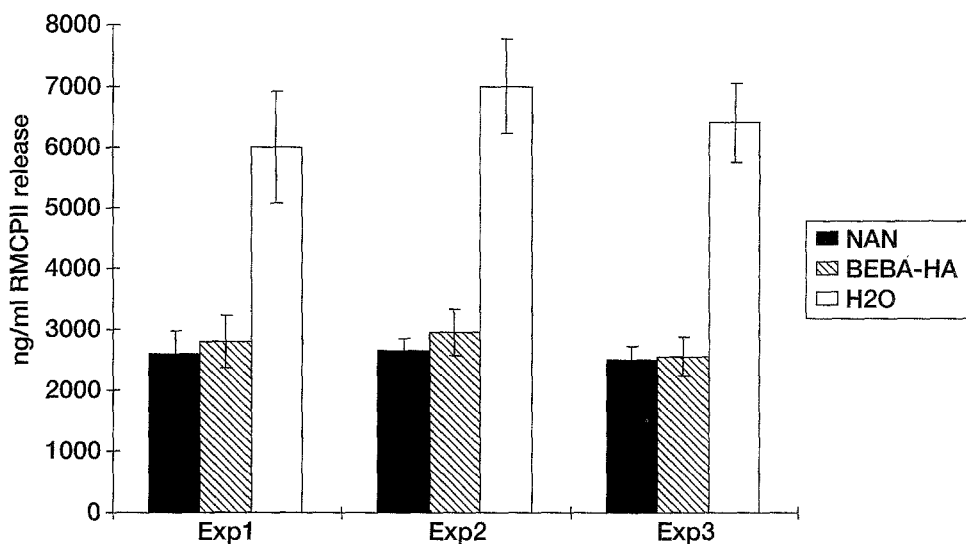


FIG. 6. In vivo suppression of mast cell response. RMCPII release from intestinal mast cells of rats orally tolerized with NAN, BEBA-HA, or water. Serum levels of RMCPII after challenge with whey proteins are shown for three different experiments in which the same protocol was used.

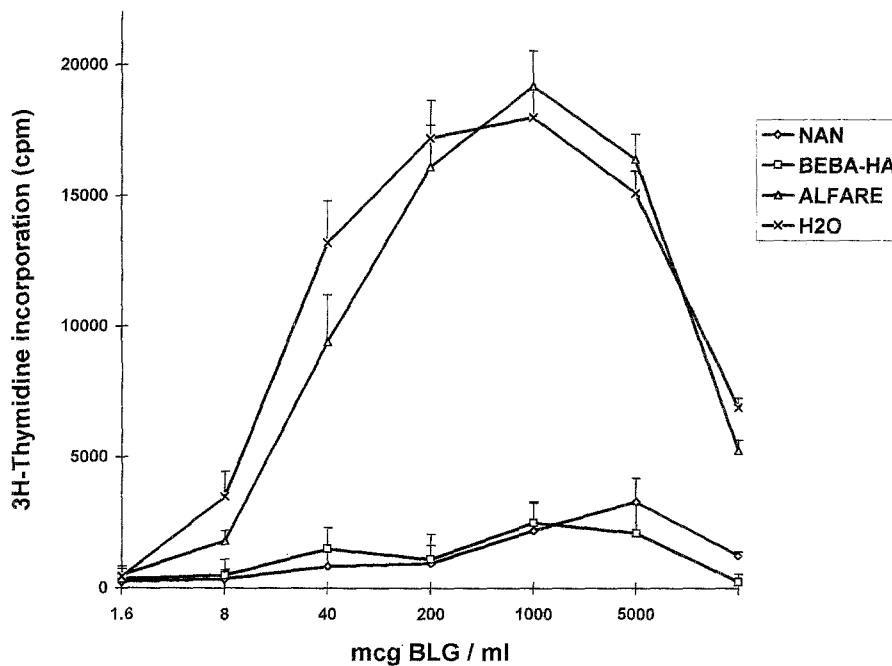


FIG. 7. Lymphocyte stimulation determined by tritiated thymidine incorporation. Lymph node lymphocytes from rats pre-fed with NAN, BEBA-HA, or ALFARE or from control rats (group given water only) were stimulated with β LG. Dose-dependent incorporation curves are shown.

These experiments show that the specific T-cell response is suppressed in rats orally tolerized by NAN or BEBA-HA feeding.

DISCUSSION

In this study we showed in a rat model that a partially hydrolyzed cow's milk formula with reduced allergenicity was able to induce specific oral tolerance to cow's

milk proteins when administered before and during allergen sensitization. In contrast, an extensively hydrolyzed cow's milk formula does not induce oral tolerance.

Cow's milk allergy is mediated primarily by specific IgE antibodies.¹¹ The suppression of IgE production is therefore a goal that may be approached in different manners. For example, we have reported earlier that the IgE production to β LG can be prevented in a rat model

by intravenous administration of a conjugate made of β LG coupled to autologous IgG.¹² However, for infants "at risk" of cow's milk allergy, the oral administration of a tolerogenic preventive formula would be the preferred choice. We have shown in this study that specific anti-cow's milk protein IgE antibodies (e.g., anti- β LG) are strongly suppressed in rats orally tolerized with a partially hydrolyzed cow's milk formula. This finding is documented both by ELISA antibody determination (over 100 times IgE titer reduction) and mast cell mediator release assays in which a total suppression of passive IgE sensitization of mast cells with sera of rats tolerized by BEBA-HA feeding was observed.

Our experiments show that as far as IgE antibody formation and the mast cell response are concerned, oral tolerance to cow's milk proteins has been induced by selected cow's milk protein peptides present in the partial hydrolysate and absent in the extensively hydrolyzed formula. This is further supported by the fact that the same doses of either the standard or the partially hydrolyzed formula induce the same levels of oral tolerance. Possible traces of contaminating intact cow's milk proteins in BEBA-HA could not explain its dose-dependent tolerizing activity, which is similar to that obtained with NAN. Otherwise, low levels of NAN would have induced the same level of antibody suppression as high levels of BEBA-HA, which has not been observed in our experiments.

We also showed that the suppression of the allergic response occurred at the intestinal level when rats were orally tolerized with the partially hydrolyzed formula. The release of the specific intestinal mast cell protease RMCP II is a good indicator of mast cell sensitization and activation.¹³ Its *in vivo* modulation by oral tolerance induction demonstrates that oral tolerizing peptides of BEBA-HA also exert their specific action at the intestinal level, a common target of the cow's milk allergic reaction.

Further evidence of the tolerizing effect of the partial hydrolysate was obtained by the isolation of a tolerogenic peptide fraction of BEBA-HA (results not shown). This peptide fraction was analyzed by high-performance liquid chromatography and SDS-PAGE. It contained no intact β LG and had a molecular weight range of 2 to 10 kd.

According to Michael,⁷ an orally administered protein must be degraded by proteolytic enzymes in the digestive system to acquire immunosuppressive properties. The cow's milk protein peptides in the partially hydrolyzed formula BEBA-HA were obtained by trypsin digestion, a process similar to physiologic *in vivo* digestion. This may explain why the tryptic peptide-containing formula (BEBA-HA) was as active as the standard formula (NAN) for oral tolerance induction in our experiments.

The doses of antigen given orally for tolerance induction are very important, because T- and B-lymphocyte modulation may be different.⁴ It was shown, for example,¹⁴ that a gavage of high doses of antigen of more than 1 mg produced suppression of delayed-type hypersensi-

tivity and IgG antibody responses, whereas low doses of 0.01 to 0.05 mg of antigen did not change the IgG response but enhanced the delayed-type hypersensitivity response. In this study we showed that modulation of the specific anti- β LG antibody response was dose-dependent in the *ad libitum* feeding protocol. High concentrations (120 mg/ml) induced strong suppression, and low concentrations (0.12 mg/ml) induced little suppression.

In addition, we showed that specific oral tolerance was also induced at the T-cell level by pre-feeding with high concentrations of NAN or BEBA-HA but not with ALFARE.

Development of oral tolerance to intact proteins has been explained by several mechanisms, which include Th anergy,^{15, 16} T_s activation,¹⁷⁻¹⁹ and bystander suppression.^{20, 21} Feeding low doses of antigen has been claimed to favor active suppression mediated by T suppressor cells, whereas higher doses favor clonal anergy.²² The discussion as to which of these mechanisms is dominant is open, although Th anergy appears to be the most accepted mechanism, at least for induction of oral tolerance to food proteins. The experiments in this study showed that oral tolerance induction with intact or partially hydrolyzed cow's milk formula was antigen-specific, and no bystander suppression against an unrelated antigen (ovalbumin) was observed. Whether the mechanisms involved for oral tolerance induction are the same for intact proteins or peptides remains to be demonstrated because peptides may be processed differently by antigen-presenting cells than whole proteins.

It is generally accepted that breast-feeding is the best choice for prevention of cow's milk allergy in infants of atopic parents. Some authors also showed a beneficial effect of maternal diet restriction during lactation on prevalence of allergy in infants,²³ whereas others found the opposite,²⁴ namely that infants breast-fed by mothers on a restriction diet had more allergies than infants of mothers not dieting. This means that tolerogenic cow's milk proteins or peptides present in the breast milk of nondieting mothers induced oral tolerance rather than sensitization in infants. Our studies showed that tolerogenic peptides are present in partially hydrolyzed formulas and not in extensively hydrolyzed ones. Therefore when breast feeding is not possible in "at risk" infants, partially hydrolyzed formulas may be more suitable than extensively hydrolyzed formulas for inducing actively oral tolerance to cow's milk proteins.

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