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Prevention of allergic sensitization to β-lactoglobulin with conjugates made of β-lactoglobulin coupled to isologous immunoglobulin G

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Abstract

TOP

We have shown that, under certain conditions, a single dose of a conjugate of β-lactoglobulin linked to isologous IgG suppresses specific antibody responses (IgE, IgG, and IgA) in rats immunized with β-lactoglobulin. This suppression is allergen specific. It also appears to be effective when animals are first immunized and then tolerized. In addition, administration of the β-lactoglobulin–IgG tolerogen abolishes mast cell mediator release. This has been shown both in vivo and with passively sensitized mast cells in vitro. We propose that the construction of a tolerogen made of whole protein allergen linked to IgG could provide a novel specific therapy for allergic reactions. (J ALLERGY CLIN IMMUNOL 1994;93:778-86.)

βLG	β-Lactoglobulin
DSS	Disuccinimidyl suberate
PHG	Phosphate-N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid-gelatin buffer

Key words: Allergy prevention, β-lactoglobulin, isologous IgG, tolerogen

Several immunologic strategies have been used either to prevent or treat allergy: chemical modification of allergens, [1] covalent linkage of allergens to nonimmunogenic carrier molecules,[2, 3] and more recently, administration of immune complexes[4, 5] or peptides alone.[6, 7] Antigen presentation appears to be the key to determining the immunologic outcome. It is known that when haptens, including nucleosides and benzyl

penicilloyl (the major determinant of penicillin), are linked to isologous IgG, they can induce specific unresponsiveness.[8] For example, benzyl penicilloyl linked to mouse IgG prevents the formation of specific IgE.[9] The recent development of a new method to link either protein or peptide to IgG[10] enabled us to test this principle for the prevention of an allergic reaction to a whole protein allergen. β-Lactoglobulin (βLG) was selected for several reasons but primarily because cow's milk allergy is very common in children.[11] Even though βLG represents only 10% of all bovine milk proteins, it is one of its most potent allergens.[12] Small amounts of βLG may pass into human milk[13] and sensitize the breast-fed infant.

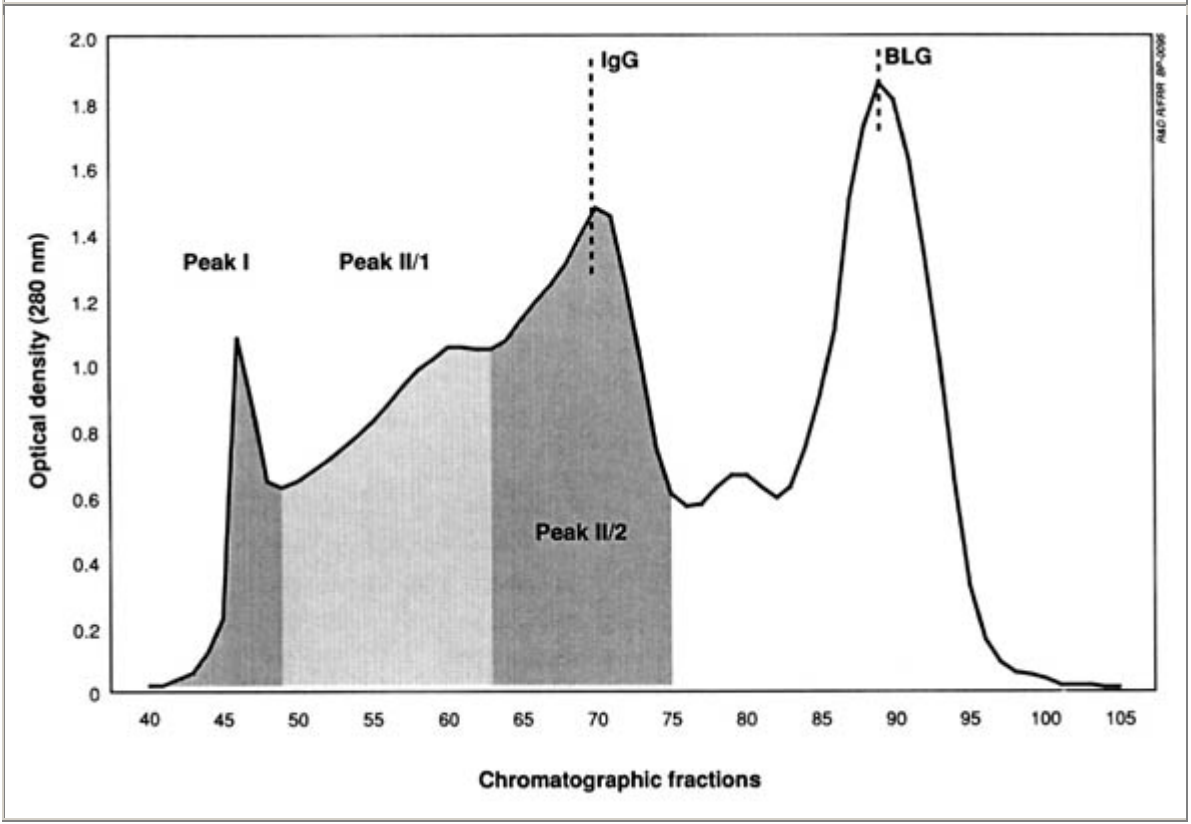
The present experiments were undertaken to determine whether one can prevent an allergic immune response in rats by a single intravenous injection of a conjugate of βLG linked to rat IgG. The results show that both the humoral response and the inflammatory manifestations of allergy are prevented.

Methods

Preparation of tolerogens

Bovine βLG was purchased from Sigma (Basel, Switzerland), purified rat IgG from ICN Immunobiologicals (Costa Mesa, Calif.), and the homobifunctional covalent reagent disuccinimidyl suberate (DSS) from Pierce Chemical Company (Rockford, Ill.). Conjugates of isologous rat IgG coupled to βLG were produced as previously described[10] but with two subsequent modifications. First, we used a 10:1 molar ratio of βLG to rat IgG as initial amounts for conjugation instead of a 1:1 ratio because we found that a 1:1 ratio failed to yield a tolerogenic conjugate. Second, the amount of DSS was also reduced by 25%. After coupling, the conjugate was fractionated by chromatography on Sephacryl S300 (Pharmacia, Uppsala, Sweden) (■Fig. 1). Fractions of 3 ml each were pooled into peaks I, II/1, and II/2. These peaks were concentrated (Amicon PM10; Amicon Inc., Beverly, Mass.) and dialyzed against NaCl 0.15 mol/L. Peak I is a polymer (high molecular weight, approximately 1000 kd), peak II/1 an IgG dimer (300 to 400 kd), and peak II/2 a monomeric IgG linked to βLG (150 to 180 kd).

FIG. 1. Sephacryl S300 separation of the βLG–rat IgG conjugate made by the DSS method. Peaks I, II/1, and II/2 are pools of fractions 40-48, 49-63, and 64-75, respectively.



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 start of the experimental period, the rats were 5 weeks old.

Experimental design

The standard experimental protocol, unless otherwise mentioned in the text, was as follows. On day 1, groups of six to seven rats were injected in the tail vein with 2 mg of βLG-rat IgG conjugate containing peak I, peak II/1, or peak II/2. Two control groups were used. One group was injected with 2 mg of a mixture of free βLG (treated with DSS) and rat IgG (1:1 molar ratio) not covalently bound to each other. The second group was not treated. Four days later, all rats were immunized by subcutaneous administration of 0.1 mg of βLG together with 0.1 mg of ovalbumin, an unrelated antigen intended to test the specificity of the immune response. Both antigens were given in Alhydrogel [$Al(OH)_3$] (Supefos Biosector A/S, Vedbaek, Denmark). Fourteen days later, which corresponds to the peak of the IgE primary response, all rats were killed and bled by cardiac puncture. Sera were kept frozen at –80° until assayed for specific IgE and IgG anti-βLG or anti-ovalbumin antibodies by ELISA. In some instances IgA antibodies were also tested. In addition, peritoneal mast cells were taken and used immediately in a tritiated serotonin release assay.

ELISA determinations of specific rat IgE and IgG antibodies

This assay was done as previously described.[14] Briefly, microtitration wells were coated with the antigen (βLG or ovalbumin) for 24 hours at 4° C, and free reaction sites were blocked with gelatin. Four washing steps with phosphate-buffered saline–Tween were performed before each successive reagent addition. After addition of test sera dilutions and incubation for 2 hours, a sheep anti-rat IgE (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-phenylenediamine. 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 a rat IgE myeloma (IR-2) and shown not to cross-react with polyclonal rat IgG. Species specificity of the peroxidase-labeled conjugates was also checked. For the determination of IgA antibodies, a commercial goat anti-rat IgA was used as a first antibody.

Analysis of the βLG to rat IgG ratio in conjugates

To measure the final molar ratio of βLG coupled to rat IgG in the tolerogenic conjugate, two immunoabsorbents were used to remove either free IgG or unbound βLG. The tolerogen (peak II/2) was initially absorbed on a protein A-Sepharose column (Pharmacia, Uppsala, Sweden). The bound material was then eluted with acidic buffer (pH 2.3), reequilibrated in phosphate-buffered saline (pH 8.0), and absorbed onto an anti-βLG immunoadsorbent (Sepharose 4B-CL CNBr-activated coupled to rat anti-βLG IgG antibodies). The acid-eluted material of this immunoadsorbent was then quantitatively analyzed by ELISA inhibition for either βLG or rat IgG. For βLG determination, a rat anti-βLG antiserum diluted 1:1000 was incubated for 1 hour at room temperature with different dilutions of βLG (standard curve) or test sample (immunoadsorbent purified βLG–rat IgG conjugates). An aliquot (0.1 ml) of this mixture was then added to βLG-coated, blocked, and washed microtiter wells. After 2-hour incubation and washing, a peroxidase-labeled anti-rat IgG was added. This was followed by incubation with the o-phenylenediamine substrate according to the previously mentioned ELISA method. For rat IgG determinations, a goat anti-rat IgG antiserum, diluted 1:1000, was preincubated for 1 hour at room temperature with different dilutions of rat IgG (standard) or test sample. An aliquot of the mixture was then added to rat IgG–coated microtiter wells and incubated for 2 hours at room temperature. After washing, a rabbit anti-goat peroxidase-labeled conjugate was added, followed by an incubation of 1 hour at room temperature and addition of o-phenylenediamine substrate as described above.

In vitro tritiated serotonin release assay

Peritoneal mast cells from experimental groups (mast cells sensitized in vivo) or mast cells of normal rats passively sensitized with sera from experimental groups (mast cells sensitized in vitro) were washed, labeled with tritiated serotonin, washed again, and triggered for mediator release by incubation with βLG dilutions

according to a published method.[14] Briefly, peritoneal lavages were obtained with Dulbecco's minimal essential medium +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–N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid–gelatin (PHG) buffer pH 7.0, cells were resuspended at 500,000 per milliliter. Ten microcuries per milliliter tritiated serotonin followed by either 1 ml of PHG (for “in vivo” sensitized cells) or 1 ml of a pool of sera from experimental groups (for “in vitro” sensitization) was added. 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 wells in which 0.05 ml of triggering agent (βLG) was mixed with 0.1 ml of sensitized mast cells. The mixture was incubated for 60 minutes at 37° C and centrifuged; 0.05 ml of the supernatant was counted for radioactivity (β-counter).

Statistics

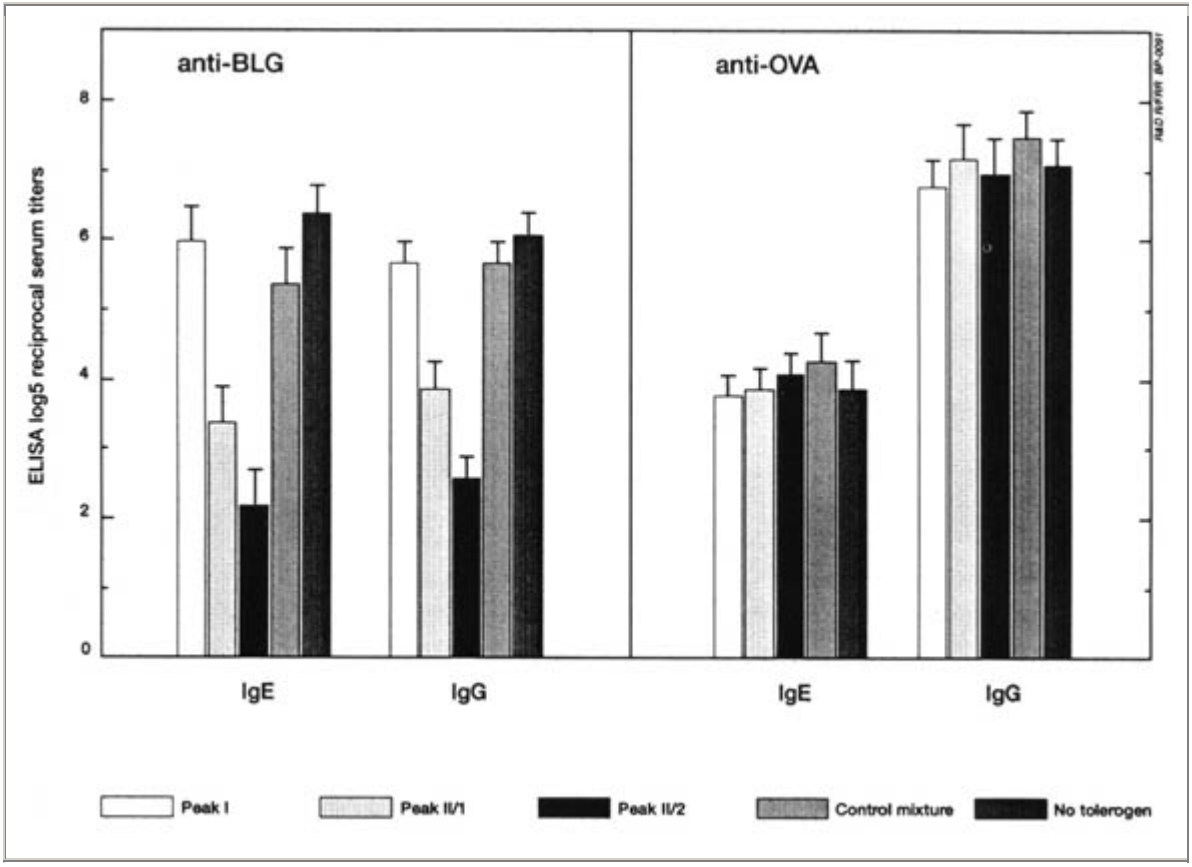
The data were tested for significance by the Fisher's exact test.

Results

Specific antibody suppression with tolerogens

Preliminary experiments were performed to determine the optimum ratio between βLG and rat IgG that yields a tolerogenic conjugate. Although an initial molar ratio of 1:1 was ineffective, a 10:1 (βLG to rat IgG) ratio consistently produced a tolerogenic conjugate. Consequently, we used this ratio in the standard experimental design described previously. A summary of four different experiments is shown in Fig. 2. It shows clearly that the IgE and IgG immune responses were depressed 500 and 100 times, respectively, in the group of rats injected with a single dose of βLG–rat IgG conjugate (peak II/2) as compared with groups injected with either peak I, the control mixture (βLG treated with DSS but unconjugated to rat IgG), or the group of control rats not pretreated. The response of the peak II/2–treated group was significantly different ($p < 0.00001$) from the responses of the other groups. Peak II/1 (the fraction of the conjugate of rat IgG dimers linked to βLG) had some suppressing activity, which was, however, lower than that obtained with peak II/2 (the rat IgG monomer fraction linked to βLG) but still significant ($p < 0.00001$) if compared with the other groups. In contrast, antibody responses to an unrelated antigen (ovalbumin) was unaffected by preventive treatments with βLG–rat IgG tolerogens (no IgE or IgG anti-ovalbumin antibody suppression), demonstrating the antigen specificity of the tolerogen-mediated immune suppression. To demonstrate further that βLG has to be covalently linked to rat IgG in order to be tolerogenic, additional controls were made by coupling βLG to itself and rat IgG to itself in the presence of DSS. Separations on Sephacryl were made as before for βLG–rat IgG conjugates, and corresponding peaks were used for intravenous prevention at the same dose (2 mg) with the standard experimental protocol. These peaks failed to suppress the anti-βLG and anti-ovalbumin antibody responses. Thus even if some βLG or rat IgG could have cross-linked in the coupling procedure, only βLG conjugated to rat IgG was tolerogenic.

FIG. 2. Antibody suppression with tolerogens. ELISA (log 5) titers of IgE and IgG anti-βLG or anti-ovalbumin antibodies in sera from peak I, peak II/1, peak II/2, rats injected with control mixture, or untreated rats.



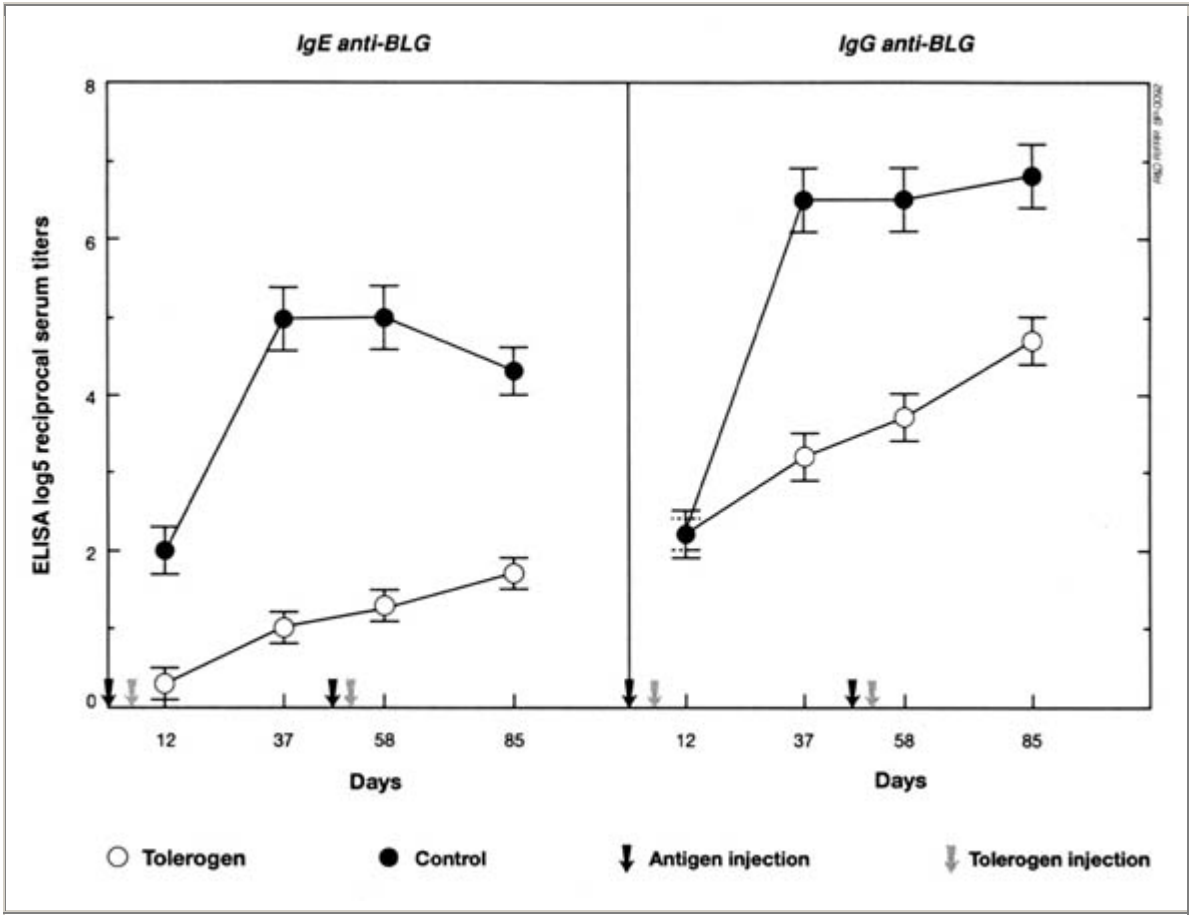
In addition to IgE and IgG suppression, IgA antibodies of rats with induced tolerance were also suppressed. Rats in which tolerance was induced with the βLG-rat IgG conjugate (peak II/2) had an IgA anti-βLG titer at a log 5 titer of 2.5 ± 0.4 compared with a titer of 5.5 ± 0.5 for the control (untreated) group of rats ($p = 0.001$).

To study the duration of the suppression, the animals were challenged at 1 month instead of 4 days after intravenous treatment. The group that received a single injection of tolerogen was still suppressed: the IgE anti-βLG log 5 titer was 3.8 ± 0.5 compared with the control group (in which tolerance was not induced), which had a titer of 6.4 ± 0.5 ($p = 0.004$). The IgG titer was at 4.4 ± 0.4, which was significantly different ($p = 0.004$) from the control group titer (6.6 ± 0.5).

Suppression of antibodies after immunization

To determine whether the same dose of tolerogen could be effective not before but after immunization, a longitudinal experiment was carried out with two groups of rats immunized with a low dose (10 µg) of βLG and ovalbumin in Al(OH)3 on days 1 and 47 of the experimental protocol. One group received a single injection of 2 mg of tolerogen 4 days after the primary and secondary immunizations, whereas the second group did not. Animals were bled on days 12, 37, 58, and 85. The results (Fig. 3) show clearly that both IgE and IgG antibody responses were suppressed. However, the diminution of IgE appears to be more profound than that of IgG both in the primary and the secondary responses. Except on day 12, antibody titers were significantly different ($p = 0.001$) between the tolerogen-treated and the control groups. Again, this was antigen-specific because tolerogen administration failed to affect the antibody response to ovalbumin: similar anti-ovalbumin titers were found in the tolerogen-treated and control groups. For example, the IgE anti-ovalbumin log 5 titer of the primary response (day 37) was 4.2 ± 04 in the tolerogen-treated group and 4.5 ± 05 in the control group.

FIG. 3. Duration of antibody suppression. Time-dependent IgE and IgG anti-βLG titers in sera of rats with induced tolerance or control rats after primary and secondary challenges with βLG.



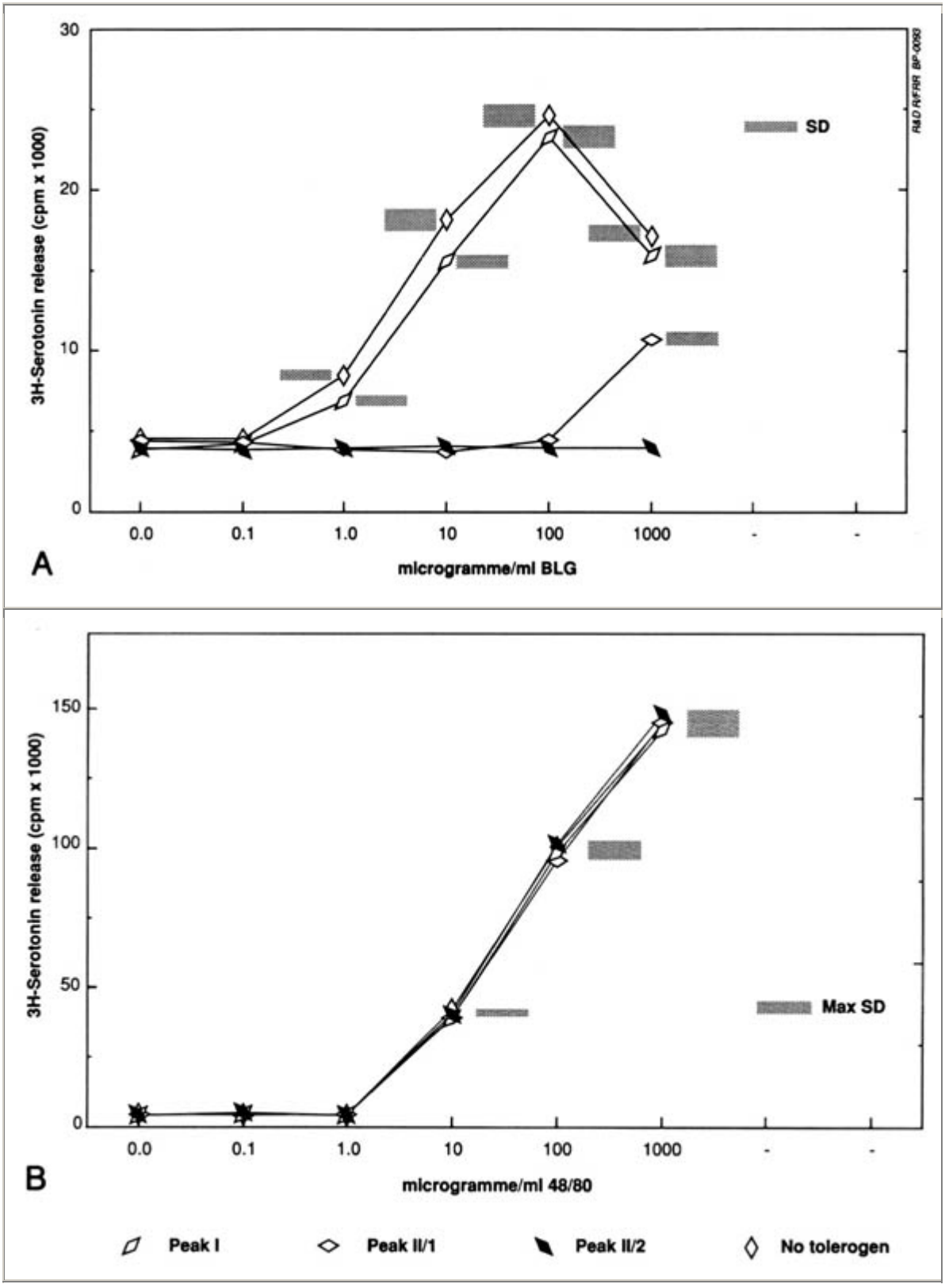
Determination of the βLG to rat IgG molar ratio of the tolerogen

To produce the tolerogens, we used an initial βLG to rat IgG molar ratio of 10:1 before adding the coupling reagent (DSS). Consequently, it was important to determine the actual binding ratio of the peak II/2 conjugate obtained after the conjugation reaction with DSS and chromatography by gel filtration. As described previously, two immunoadsorbents were used: the first was protein A-Sepharose to remove the unbound βLG, and the second was an anti-βLG Sepharose to remove the rat IgG not conjugated to βLG. The end product, which contained only βLG–rat IgG conjugates was then analyzed by ELISA inhibition for its content of βLG and rat IgG. We found that the molar ratio of βLG to rat IgG was 1:1. Because we injected 2 mg of conjugate (peak II/2, containing monomeric IgG), the amount of βLG covalently linked was 0.21 mg.

Effect of tolerogens on mast cell reactivity

Initially, it was determined whether pretreatment with tolerogens could modulate the specific release of mediators from mast cells of sensitized rats. It was found (Fig. 4, *A*) that peritoneal mast cells from rats in which tolerance was induced with peak II/2 failed to release prelabeled tritiated serotonin when specifically triggered with βLG. Rats in which tolerance was induced with peak II/1 had a low level of sensitized mast cells, which were stimulated for mediator release only by high amounts of βLG (1 mg/ml). In contrast, mast cells from rats treated with peak I or those from untreated rats showed a typical dose-dependent tritiated serotonin release curve when triggered with βLG. When the triggering of these target mast cells was made with ionophore compound 48/80, a nonspecific mast cell triggering agent, no difference of reactivity could be observed between the different experimental groups (Fig. 4, *B*). This demonstrated the functional integrity of mast cells from rats rendered unresponsive.

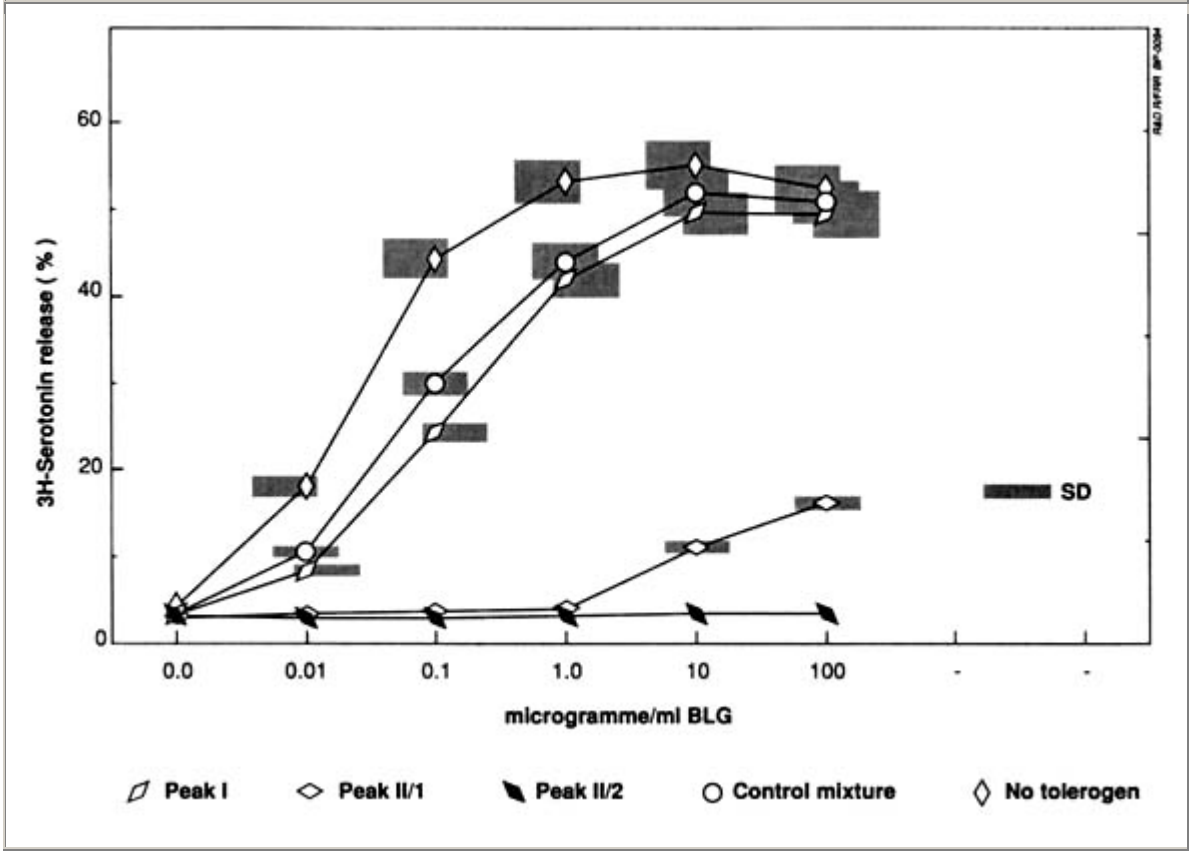
FIG. 4. Tritiated serotonin release from in vivo sensitized mast cells of rats treated with peak I, peak II/1, and peak II/2 or untreated rats. Triggering was done with either βLG **(A)** or compound 48/80 **(B)**.



It seemed logical to assume that mast cells from animals having a low level of IgE anti-βLG in their serum (rats with induced tolerance) were weakly, if at all, sensitized in vivo with specific IgE. To test this idea, normal rat mast cells were sensitized with sera of either rats with induced tolerance or control rats. Fig. 5 shows that sera of rats of which tolerance was induced in vivo with the conjugate (peak II/2) failed to sensitize normal rat mast cells for tritiated serotonin release when triggered with βLG. Similar release curves to those seen with in vivo sensitized mast cells were obtained when passive sensitization was performed with sera from the different experimental groups. Therefore rats with successfully induced tolerance have low

levels of residual IgE anti-βLG reaginic antibodies (as shown by ELISA), which are inefficient for active in vivo or passive in vitro sensitization of target mast cells.

FIG. 5. Tritiated serotonin release from normal mast cells passively sensitized in vitro with sera from rats treated with peak I, peak II/1, peak II/2, and control mixture or sera from untreated rats. Triggering was done with βLG (0.01 to 100 µ/ml), and tritiated serotonin release is expressed as percent of total release obtained with $HC10_4$ 6%.



It was also of interest to determine whether the tolerogen by itself was able to trigger mediator release. To examine this, normal rat mast cells were passively sensitized with rat anti-βLG IgE antibodies followed by triggering with either intact βLG or βLG conjugated to rat IgE (peak II/2). We found that there was a 10-fold reduction in triggering capacity of the same amount of βLG, linked to IgG, as compared with βLG alone. For example, 45% of total tritiated serotonin release was obtained by triggering with either 0.1 µg/ml βLG or 10 µg/ml peak II/2 (which contains 1 µg/ml βLG).

Discussion

Our major observation is that one can suppress the IgE, IgG, and IgA antibody primary immune response to βLG, a potent cow's milk allergen, by a single intravenous dose of βLG conjuated to rat IgG. This is allergen-specific, because the immune response to an unrelated antigen (ovalbumin) is unaffected. In addition, pretreatment with the tolerogenic protein conjugate abolishes serotonin release by the rat mastocytes sensitized either in vivo or in vitro. βLG was selected not only because it is a potent cow's milk allergen in children but also because it resists acid digestion in the stomach; minute amounts of the intact protein can pass into the circulation of the secretory mammary gland of the mother and, through human milk, sensitize the breast-fed infant.[15] In some instances, it can cause anaphylactic shock.[16] This protein therefore is an ideal candidate with which to test the hypothesis that one can transform a protein antigen into a tolerogen by covalent linkage of the whole protein to isologous IgG.

At least two experimental conditions appear to be critical in construction of a tolerogenic protein conjugate: the degree of substitution of βLG linked to IgG and the physical form of the conjugate. An initial molar ratio

of 10 βLG to 1 IgG is necessary to yield a consistently tolerogenic protein conjugate. To analyze the final molar ratio of the conjugate after covalent linkage with DSS and chromatography on Sephacryl, two immunoabsorbents were used to remove either free IgG or βLG aggregates. The remaining conjugate was then tested by ELISA inhibition. We found that the degree of substitution of βLG linked to IgG was 1:1. Because we injected a single dose of 2 mg of protein conjugate, it means that about 200 µg of βLG linked to rat IgG is sufficient to suppress the primary immune response. This calculation is based on a molecular weight of 18 kd for βLG because at pH 8.3 most of the βLG is in a single, monomeric polypeptide chain.[17] Several control studies were performed, which showed that only the conjugate of monomeric rat IgG linked to βLG in a 1:1 molar ratio (peak II/2) was a potent tolerogen. High molecular weight conjugate (peak I) was inactive, and although peak II/1 (dimeric IgG coupled to βLG) has a specific suppressive activity, this could in part result from contamination by the monomeric conjugate in these fractions. The mixture of free βLG and IgG was unable to induce either antibody or mast cell suppression. In addition, neither IgG nor βLG, cross-linked to themselves by the homobifunctional reagent (DSS), was active. Consequently, as shown in other experimental models, the antigen has to be covalently linked to IgG in order to be tolerogenic.[18]

The suppressing activity of the tolerogen seems to be lasting because challenge of the animals 1 month after administration of a single dose of tolerogen still resulted in a reduction of antibody formation. Of greater significance is the observation that the administration of tolerogen to sensitized animals is effective both in the primary and the secondary immune responses. This suggests that under appropriate experimental conditions, this approach may be used not only to prevent but also to treat an allergic reaction. The results clearly show that suppression was obtained in the B-cell compartment because antibody responses of different isotypes to βLG were diminished. It is interesting to note that IgE appears to be more susceptible to tolerance induction than IgG antibody formation. For example, in the primary response IgE antibody formation was reduced 500 times, whereas IgG was reduced 100 times. This was also apparent in sensitized animals, particularly in the secondary response. It is known that unaggregated monomeric IgG is a strong tolerogen, and it has been recently shown to induce tolerance in T_{H1} but not T_{H2} cells.[19] However, IgE antibody responses are favored by T_{H2} cells,[20] suggesting that the tolerogenic conjugate might affect both T_{H2} and T_{H1} cells. The cellular mechanism by which the monomeric conjugate is the most active is unknown. However, with haptens linked to IgG, both T and B cells are involved either in animals “in vivo” or in human “in vitro” studies.[21, 22, 23]

Mast cells are the target cells in immediate hypersensitivity. It was of interest therefore to determine whether the IgE-mediated reactivity of both in vivo and in vitro sensitized cells was modulated by the intravenous administration of tolergen. We found that mast cells obtained from rats treated with the tolerogen failed to release mediator when triggered with βLG. In contrast, these cells released the same amount of serotonin when triggered by an ionophore (compound 48/80), thus demonstrating that these cells were viable and functionally active. It also indicated that the residual IgE antibody to βLG still present in the sera of the animals suppressed by the tolerogen injection was unable to sensitize the mast cells for mediator release. This was further demonstrated with the use of mastocytes from naive rats passively sensitized with sera from rats pretreated with tolerogen.

The observation that the tolerogen (peak II/2) by itself can trigger mediator release from passively sensitized mast cells indicates that even linked to rat IgG, βLG keeps a part of its IgE-specific allergenic epitopes functionally active. The triggering activity of the conjugate is, however, reduced if compared with intact βLG, indicating that steric hindrance of βLG linked to rat IgG probably plays a role. It is conceivable that this modification of the spatial conformation of βLG in the tolerogenic conjugate may also be important for inducing unresponsiveness. Further experiments are necessary to demonstrate whether or not allergenic and tolerogenic epitopes are on different sites of the βLG molecule.

Many different approaches have been recently used to suppress allergic reactions, including administration of chemical modified allergens and antibody complexes and administration of peptides alone.[1-7] Our approach is different not only because a whole potent protein allergen has been transformed into a tolerogen by covalent linkage to IgG but also because it does not raise another immune response, such as IgG-blocking antibody. It appears instead to suppress all antibodies of different isotypes including IgA. In addition, in contrast to polyethylene glycol–modified allergen,[24] it appears to be effective in sensitized animals. This observation needs to be confirmed and extended not only to food but also to aeroallergens. We hope that this new approach will provide a novel way to prevent and possibly treat allergic and autoimmune diseases.

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