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Immunology

Immunological tolerance to allergenic protein determinants: A therapeutic approach for selective inhibition of IgE antibody production

[ragweed antigen E–poly(D-Glu,Lys)/ovalbumin–poly(D-Glu,Lys)/IgE antibodies/human allergies/immunotherapy]

FU-TONG LIU AND DAVID H. KATZ

Department of Cellular and Developmental Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037

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ABSTRACT **Administration of stable conjugates prepared by coupling protein antigens such as ovalbumin or antigen E of ragweed extract to the synthetic random copolymer of D-glutamic acid and D-lysine (D-GL) is effective in inducing a state of long-lasting, antigen-specific immunological tolerance in experimental animals. A striking aspect of the tolerance induced by protein–D-GL conjugates is the remarkable selectivity of the tolerance for antibody responses of the IgE class. Protein–D-GL conjugates of either type were capable of inducing such tolerance both in unsensitized and in previously sensitized animals when administered in appropriate doses. Comparable doses of unconjugated proteins were likewise capable of suppressing IgE antibody production, although the duration of suppression in these cases was significantly less than that observed with protein–D-GL conjugates. If such conjugates act in man as they do in experimental animals, they could be of great value as therapeutic agents in selectively diminishing IgE antibody production while sparing antibody production in the IgG class.**

In this report, we describe experimental conditions that are successful in inducing specific immunological tolerance, selective to the IgE antibody class, to the major sensitizing determinants on two complex proteins, ovalbumin (OVA) and ragweed antigen E (AgE). This has been accomplished by administering suitable doses of conjugates prepared by covalently coupling either OVA or AgE to the synthetic random copolymer of D-glutamic acid and D-lysine (D-GL).

This work represents an extension of studies conducted in this laboratory over the past few years that have demonstrated (1, 2) and characterized (refs. 1–6; reviewed in refs. 7 and 8) a system of prolonged hapten-specific B cell (bone-marrow derived) tolerance induced in either unsensitized or previously sensitized experimental animals by administering the relevant hapten–D-GL conjugate in appropriate doses. This method has proven to be extremely effective in several animal species (7–10) in inducing tolerance in the B lymphocyte precursors of antibody-forming cells of the IgM, IgG, and IgE (9, 10) antibody classes specific for the 2,4-dinitrophenyl nucleoside (11), and benzylpenicilloyl (12) haptens.

Whereas the previously investigated models of hapten-specific tolerance have significant therapeutic potential for allergic disorders in which sensitivities to known haptenic determinants are involved, a large variety of IgE-mediated human allergic diseases reflect hypersensitivities to allergenic determinants on complex protein antigens. It was of substantial importance, therefore, to ascertain whether similar tolerogenic effects could be induced by administering protein–D-GL conjugates. The first hurdle to overcome in this respect pertained to the chemical methodology that would allow preparation of these conjugates in such a way that a stable linkage existed between the respective protein and D-GL molecules, there was ample retention of the native antigenic determinants on the protein portion of the conjugate following exposure to the coupling reaction, and the desired protein–D-GL conjugate could be obtained in pure form devoid of any detectable contaminating protein–protein aggregates or uncoupled protein monomers. We have recently developed suitable technology that meets the aforementioned criteria, the details of which are described elsewhere (13). The biological efficacy of such protein–D-GL conjugates in inducing the desired immunologically-specific tolerance has been proven, as reported herein.

MATERIALS AND METHODS

Chemicals. D-GL(D-Glu^{60}D-Lys^{40}, M_r 63,700), hen egg OVA (five times recrystallized), ragweed extract, and AgE were all obtained from Miles.

Preparation of Protein–D-GL Conjugates. The general methodology for the preparation, purification, and characterization of protein–D-GL conjugates has been reported elsewhere (13). The detailed procedures for the preparation of OVA–D-GL are described therein. The detailed procedures for the preparation of AgE–D-GL are unpublished. Briefly, 20 mg (540 nmol) of AgE was mixed with 4.2 mg (13.5 μmol in 200 μl of dimethylformamide) of maleimidobenzoyl-*N*-hydroxysuccinimide ester in 2.0 ml of 0.01 M phosphate buffer, pH 7.0, and the product (maleimidobenzoyl-AgE) of the subsequent reaction was isolated by gel filtration chromatography on Sephadex G-25, as described for maleimidobenzoyl-OVA (13). AgE–D-GL was prepared by mixing maleimidobenzoyl$_{3.6}$-AgE (16 mg, 433 nmol in 3.6 ml of 0.1 M phosphate buffer, pH 6.0) with thiolated SH$_{1.9}$-D-GL–biotin$_{4.5}$ (30.6 mg, 480 nmol, containing trace quantity of ^{125}I-labeled molecules, in 3.13 ml of phosphate-buffered saline containing 0.01 M EDTA) and isolated by affinity column chromatography on avidin-Sepharose, as described for OVA–D-GL (13). OVA–D-GL contained a ratio of OVA to D-GL of 0.6–0.7:1, and the modified protein retained 70–80% of its antigenicity. AgE–D-GL contained a ratio of AgE to D-GL of 0.5:1 and retained 10–20% of its antigenicity. In both cases, antigenicity was measured by degree of reactivity of the conjugate with specific anti-OVA or anti-AgE antibodies.

Animals and Immunization. CAF$_1$ mice (8–12 weeks old unless otherwise specified) were obtained from the Jackson Laboratory, and were immunized and challenged intraperitoneally (i.p.) with 10 μg of OVA or 5 μg of AgE (or ragweed extract) adsorbed on Al(OH)$_3$ gel (alum, 4 mg or 2 mg) as de-

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Abbreviations: AgE, antigen E of ragweed extract; B, bone marrow-derived; D-GL, copolymer of D-glutamic acid and D-lysine; i.d., intradermal; i.p., intraperitoneal; i.v., intravenous; OVA, ovalbumin; T, thymus-derived.

scribed (14) and according to experimental protocols given in *Results*.

Measurement of Antibodies. The concentration of IgE anti-OVA or anti-AgE antibodies were quantitated by passive cutaneous anaphylaxis reactions in rats as described (14, 15), with titers presented as reciprocals of highest dilutions yielding positive reactions.

The IgG antibody activity of mouse anti-OVA antisera was measured by solid-phase radioimmunoassay using ^{125}I-labeled rabbit anti-mouse Fab (16). That of mouse anti-AgE antisera was determined by a double antibody method using ^{125}I-labeled AgE and a rabbit anti-mouse serum.

RESULTS

OVA-D-GL Induces Persistent Tolerance in the IgE, but Not the IgG, Antibody Class in CAF$_1$ Mice. The three groups of normal CAF$_1$ mice were injected intradermally (i.d.) and intravenously (i.v.) with four doses, administered at 2-day intervals, of either OVA-D-GL containing 250 μg of total D-GL (100 μg of conjugated OVA), 100 μg of unconjugated OVA, or a mixture of 250 μg of D-GL plus 100 μg of unconjugated OVA. One day after the fourth dose, these mice and a group of untreated control mice were primarily immunized with 10 μg of OVA plus alum (day 0). On day 15, mice were treated again and then secondarily challenged with 10 μg of OVA plus alum.

As shown in Fig. 1 *lower*, control mice developed very good primary and secondary anti-OVA IgE antibody responses. Mice pretreated and later secondarily treated with OVA-D-GL failed to produce detectable anti-OVA IgE antibody responses at any time during the period of observation. This unresponsiveness persisted for a long time, even after a third challenge with the sensitizing dose of OVA administered 45 days after the second treatment with OVA-D-GL. Groups of mice which were treated with either OVA or a mixture of OVA plus D-GL also displayed suppressed IgE antibody responses. The pattern of unresponsiveness in these latter two groups was, however, significantly different from that manifested by mice treated with OVA-D-GL. In both cases the suppression of IgE antibody production was transient and followed by a rebound production of anti-OVA IgE antibodies at levels that were at times higher than those produced by the untreated control mice. A particularly pertinent contrast in the relative effectiveness of these different modes of treatment is illustrated by the ability of mice treated with a mixture of OVA plus D-GL to develop significant IgE anti-OVA responses after tertiary antigenic challenge administered relatively late in the course (day 66), whereas mice treated with OVA-D-GL were totally unresponsive at this time.

In contrast with the clear effectiveness of OVA-D-GL in inducing unresponsiveness in the IgE antibody class, this treatment failed to diminish anti-OVA antibody responses of the IgG class and, moreover, actually appeared to heighten the IgG responses (Fig. 1 *upper*). This was true not only of mice treated with OVA-D-GL, but also of those mice treated with either unconjugated OVA or a mixture of D-GL plus OVA. Note that these treated mice produced higher levels of IgG anti-OVA antibodies than the corresponding untreated control mice, particularly during the early stages of observation.

Comparable results were obtained in a separate experiment of similar design by using AgE-D-GL as a means for abolishing IgE antibody responses specific for AgE (data not shown).

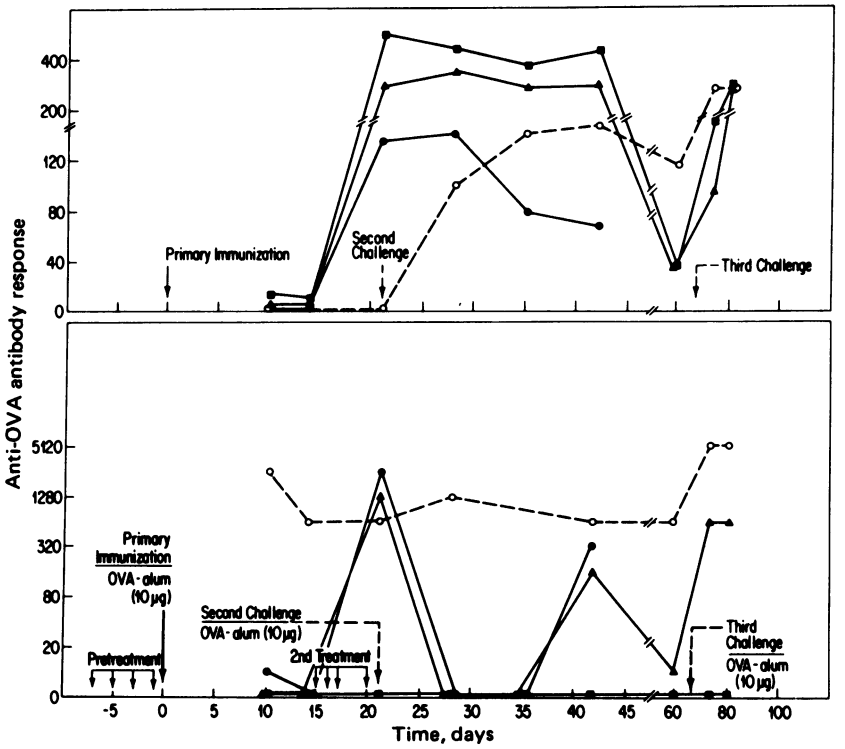

FIG. 1. OVA-D-GL induces persistent tolerance in the IgE, but not the IgG, antibody class in CAF$_1$ mice sensitized repeatedly with OVA in alum. Normal CAF$_1$ mice were either not treated (○) or treated with unconjugated OVA (100 μg) (●), a mixture of unconjugated OVA (100 μg) plus D-GL (250 μg) (▲), or OVA-D-GL (250 μg of D-GL containing 100 μg of OVA) (■). Pretreated mice were injected four times, receiving the dose indicated each time. Doses were administered i.d., i.v., i.v., and i.d. on alternating days. One day after the fourth dose, all mice were primarily immunized with 10 μg of OVA in 4 mg of alum. The second treatment was administered on days 15 (i.p.), 16 (i.d.), 17 (i.p.), and 20 (i.d.). A secondary challenge was carried out on day 21 with 10 μg of OVA in 2 mg of alum. Tertiary challenge was carried out on day 66 in the same manner (all immunizations were given i.p.). Serum IgE (*Lower*) and IgG (*Upper*) anti-OVA antibody responses of groups of three mice bled on various days after primary immunization, as indicated, are illustrated.

Induction of Tolerance in the IgE Antibody Class by Administration of OVA–D-GL to CAF_1 Mice Previously Sensitized to OVA. Two groups of untreated mice were primarily sensitized with 10 μg of OVA plus alum. Fifteen days later, one group was injected i.d. and i.v. with four doses of OVA–D-GL; the second group was not treated. One day after the last dose of OVA–D-GL, both groups were secondarily challenged with 10 μg of OVA plus alum.

As shown in Fig. 2 *lower*, immediately after treatment with OVA–D-GL, and just prior to secondary challenge, such treated mice displayed higher levels of IgE anti-OVA antibodies than the untreated controls. However, in contrast to the untreated group, which developed good secondary responses, the OVA–D-GL-treated mice displayed a sharp drop in their IgE anti-OVA antibody levels. These depressed responses in such treated mice persisted for 15–18 days, after which their IgE antibodies rose briefly to normal levels and then subsided to 50% of control titers by day 59. At that time, this group was treated a second time and then given a third challenge with 10 μg OVA plus alum. Unlike the untreated control mice, which developed substantial tertiary responses after such challenge, the OVA–D-GL-treated mice not only failed to respond but, to the contrary, actually displayed diminution of their IgE anti-OVA antibodies to undetectable levels.

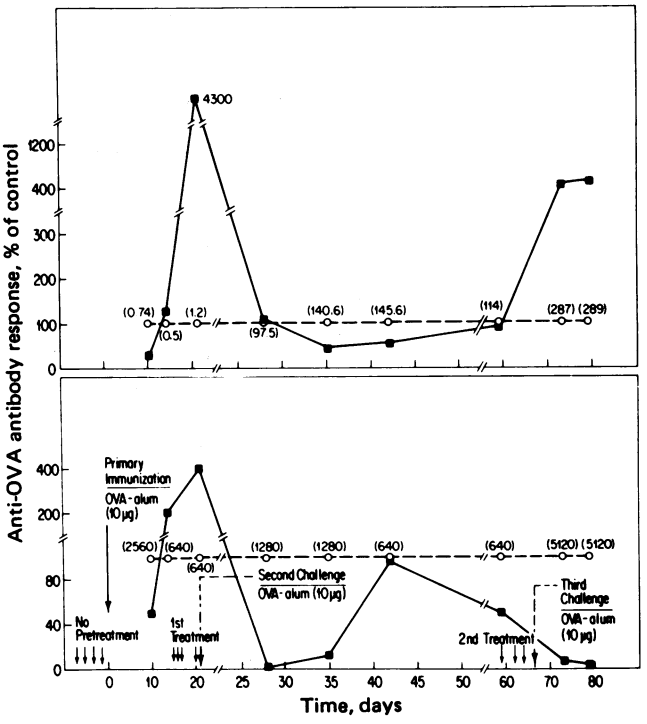

FIG. 2. Induction of tolerance in the IgE antibody class by administration of OVA–D-GL to CAF_1 mice previously sensitized to OVA. Normal CAF_1 mice were primarily immunized with 10 μg of OVA in 4 mg of alum on day 0. Two weeks later, one of these groups was treated with OVA–D-GL (250 μg of D-GL containing 100 μg of OVA) administered on days 15 (i.d.), 16 (i.v.), 17 (i.v.), and 20 (i.d.) (250 μg per injection). On day 21, this group and the untreated control mice were secondarily challenged with 10 μg of OVA in 2 mg of alum. On day 59, the group of OVA–D-GL-treated mice was subjected to a second treatment regimen with OVA–D-GL administered on days 59 (i.p.), 62 (i.d.), 64 (i.p.), and 66 (i.d.) in the same dose given for the initial treatment. Also on day 66, both groups were given a tertiary challenge of 10 μg of OVA in 2 mg of alum. Serum IgE (*Lower*) and IgG (*Upper*) antibody responses of the treated mice (■) are represented as percentage of the response developed by the untreated control group (○), with the actual antibody levels of the controls indicated in parentheses above or below each data point. Each group consisted of three mice.

The selective nature of tolerance induction for antibodies of the IgE class was again observed in this experiment. As shown in Fig. 2 *upper*, the anti-OVA IgG antibody response of the treated group was 43-fold higher than that exhibited by the untreated controls after the first treatment with OVA–D-GL. This marked hyperresponsiveness in the IgG class subsided such that the OVA–D-GL-treated mice produced comparable levels of IgG antibodies to those of the control group after subsequent secondary challenge. However, after the second treatment with OVA–D-GL (day 59), IgG antibody production was again enhanced in the treated mice.

AgE–D-GL Induces Tolerance in the IgE Antibody Class when Administered to CAF_1 Mice 1 Year After Initial Sensitization with AgE. Although the preceding experiments demonstrate the efficacy of protein–D-GL conjugates in inducing specific immunological tolerance in either unsensitized or previously sensitized mice when analyzed in acute circumstances, we wished to ascertain how effective this approach would be in circumstances that more closely approximated a clinical allergy problem. Our rationale was, therefore, to sensitize mice, in this case with AgE, and then let them rest for a period of 1 year before subjecting them to any additional manipulation. After this prolonged interval, certain mice would be treated, others not, and determinations would be made of their relative capacities to develop specific antibody responses after subsequent challenge with AgE. The results of such a study are summarized in Fig. 3.

CAF_1 mice were exposed to a low dose of whole body ionizing irradiation shortly prior to primary sensitization with 10 μg of ragweed extract plus alum. The reason for exposing such mice to low doses of irradiation pertains to previous investiga-

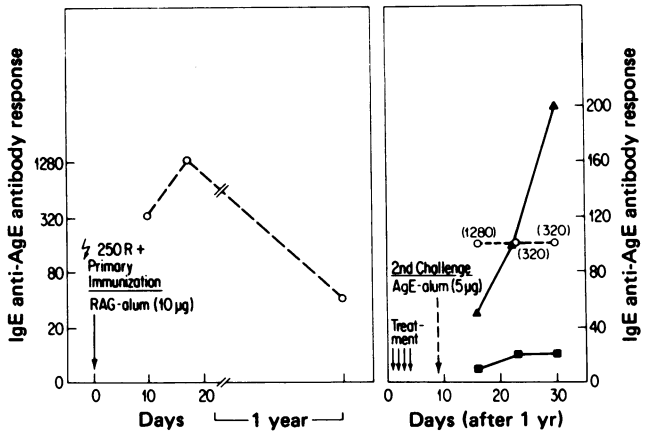

FIG. 3. AgE–D-GL induces tolerance in the IgE antibody class when administered to CAF_1 mice 1 year after initial sensitization with AgE. Normal CAF_1 mice were exposed to 250 R of whole body x-irradiation shortly prior to primary immunization with 10 μg of ragweed extract in 4 mg of alum (1 R = 2.58×10^{-4} C/kg). All mice were bled on days 10 and 20 after sensitization, and the levels (passive cutaneous anaphylaxis) of IgE anti-AgE antibodies are illustrated in *Left*. These mice were then left to rest for an interval of 1 year, at the end of which they were bled for determinations of residual levels of IgE anti-AgE antibodies (*Left*). These mice were then divided into three groups of which two were given four injections, each consisting of either unconjugated AgE (75 μg, i.d., i.p., i.p., and i.p.) (▲) or AgE–D-GL (250 μg of D-GL containing 75 μg of AgE, i.d., i.p., i.p., and i.p.) (■). The injections were given at daily intervals. Five days after the final injection, these two treated groups and a third group of untreated control mice (○) were then challenged with 5 μg of AgE in 4 mg of alum. The IgE anti-AgE antibody responses of groups of three mice each are presented (*Right*) as percentage of the control passive cutaneous anaphylaxis response developed by untreated mice, with the actual control values illustrated in parentheses above or below the corresponding data point after secondary challenge.

tions in this laboratory that demonstrated that such manipulations resulted in substantial enhancement of the magnitude of IgE antibody production after sensitization with any number of antigens (17, 18). As shown in Fig. 3 *left*, this immunization regimen resulted in very good primary IgE anti-AgE antibody responses. After a 1-year interval of rest, all of these mice were bled to determine the magnitude of specific anti-AgE IgE antibodies detectable in their serum at that time. It is of interest to note that all mice so tested had detectable IgE antibodies, even though they had not been subsequently exposed to AgE during the 1-year rest period.

Mice producing the lowest titers of IgE antibodies (passive cutaneous anaphylaxis titer = 40) were then divided into three groups. Two groups were injected i.d. and i.p. with four doses of either AgE–D-GL containing 250 μg of D-GL (75 μg of conjugated AgE) or 75 μg of unconjugated AgE. A third group was left untreated as controls. Five days after the last dose, all mice were secondarily challenged with 5 μg of AgE plus alum. As shown in Fig. 3 *right*, untreated control mice developed excellent secondary IgE antibody responses, which peaked 7 days after secondary challenge. Mice treated with unconjugated AgE, although manifesting 50% lower responses than untreated controls on day 7, produced IgE anti-AgE responses either comparable to or 2-fold higher than those of controls later in the response. In marked contrast, those mice treated with AgE–D-GL displayed a marked inability to develop anything other than very meager AgE-specific IgE responses.

DISCUSSION

These results demonstrate the successful induction of specific immunological tolerance to two different protein antigens, OVA and AgE, in both unsensitized and previously sensitized experimental animals, and the tolerance is selectively confined to responses of the IgE antibody class. Such tolerance resulted from the administration of appropriate doses of the respective protein–D-GL conjugates. Studies currently underway in our laboratory have documented the absolute antigen specificity of the tolerant state induced with one or the other of the two protein–D-GL conjugates employed here and, moreover, that the mechanism of unresponsiveness obtained with protein–D-GL conjugates does not involve the participation of detectable active suppressor cells (unpublished observations).

Two points about these findings are worthy of particular comment. First, it is obvious from these data that IgE antibody responses could be suppressed not only by administration of protein–D-GL conjugates, but also by administering comparable doses of unconjugated protein alone. It should be emphasized, however, that the patterns of IgE antibody production following treatment in each of these two ways were significantly different. Thus, in general, administration of unconjugated protein suppressed IgE production effectively, but only transiently; in one case of particular note, namely when treatment was administered after a 1-year interval of rest following initial sensitization (Fig. 3), administration of unconjugated protein had only marginal inhibitory effects on the specific response, and this effect was shortly followed by a marked "booster" effect on the specific IgE response. Administration of protein–D-GL conjugates, on the other hand, resulted in an inhibition of IgE antibody production that persisted for long periods of time, even after repeated exposure to the sensitizing antigen. We have no information at the present time about whether the mechanism of tolerance induced by these two different methods is qualitatively the same or different.

The second point worth emphasizing is the remarkable selectivity of unresponsiveness observed in these studies. IgE antibody responses were markedly diminished whereas, concomitantly, specific IgG antibody responses to the same determinants tended to be increased, irrespective of whether protein–D-GL conjugates or unconjugated proteins were administered to test mice. This represents a major difference between the protein–D-GL system and the hapten–D-GL systems studied earlier; in the latter systems, it was clear that antibody responses of all immunoglobulin classes were susceptible to tolerance induction after exposure to hapten–D-GL conjugates (7). Quite frankly, we have no data at present that would help to explain the selectivity of protein–D-GL conjugates for responses of the IgE class, and additional studies are necessary to clarify this point. It could be, for example, that the relative concentration of protein determinants on a given D-GL molecule may determine the extent of Ig class selectivity observed. Nevertheless, it is clear that fundamental differences exist in the susceptibility to tolerance induction of the IgE and IgG antibody systems, respectively, under the conditions of the experiments reported here. Establishment of the basis for this difference will be of great significance in furthering our understanding of regulatory control of these two antibody classes.

In the hapten–D-GL tolerance models, substantial evidence has been obtained demonstrating the rapid and irreversible inactivation of B lymphocytes specific for the hapten employed after brief exposure to the conjugate, possibly by disturbance of normal membrane machinery (7, 8). The mechanism of tolerance induction by protein–D-GL conjugates has yet to be established. The conjugate may be acting directly on B lymphocytes, notably those of the IgE class, on protein-specific T lymphocytes (thymus-derived) (of either helper or suppressor type, or both), or on both B and T lymphocytes. Studies in other laboratories have recently demonstrated that antigen-specific suppressor T cells, capable of suppressing IgE antibody production, can be generated in experimental animals by administering urea-denatured antigen (19) or protein coupled to polyethylene glycol (20). In the latter study, controls for the suppressive effects of unconjugated protein were not reported, thus leaving open the possibility that the suppression obtained with protein–polyethylene glycol conjugates may be similar to that obtained with unconjugated protein alone, as demonstrated in the present study. Although inhibition of IgE antibody production by the function of antigen-specific T cells is itself important, we do not believe that the practicality of such approaches as a therapeutic modality will be far-reaching due to the transient nature of such suppression phenomena.

It should be noted that one recent report (21) claimed that dinitrophenyl–D-GL induced dinitrophenyl-specific suppressor T cells in a murine system. However, since the experimental conditions employed were not adequate for eliminating the possible carry-over of tolerogenic dinitrophenyl-D-GL molecules in the cell mixtures, this interpretation may not be valid. Nevertheless, as stated above, there is no *a priori* reason not to consider that the mechanisms of tolerance induction with hapten–D-GL and protein–D-GL conjugates, respectively, could be quite different.

The obvious implication of our results is that allergenic proteins coupled to D-GL may prove useful in man for the specific abrogation of IgE antibody responses to the relevant allergen in those IgE-mediated disorders in which the nature of the predominant sensitizing proteins are known. The fact that protein–D-GL conjugates induce *selective* inhibition of IgE antibody production, while not diminishing IgG antibody responses against the same antigen, meets criteria for ideal properties of therapeutic agents of this type for use in human allergic diseases.

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