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Severe anaphylactic reactions to glutamic acid decarboxylase (GAD) self peptides in NOD mice that spontaneously develop autoimmune type 1 diabetes mellitus

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Abstract

Background: Insulin dependent (i.e., "type 1") diabetes mellitus (T1DM) is considered to be a T cell mediated disease in which T_H1 and T_c autoreactive cells attack the pancreatic islets. Among the beta-cell antigens implicated in T1DM, glutamic acid decarboxylase (GAD) 65 appears to play a key role in the development of T1DM in humans as well as in non-obese diabetic (NOD) mice, the experimental model for this disease. It has been shown that shifting the immune response to this antigen from T_H1 towards T_H2, via the administration of GAD65 peptides to young NOD mice, can suppress the progression to overt T1DM. Accordingly, various protocols of "peptide immunotherapy" of T1DM are under investigation. However, in mice with experimental autoimmune encephalomyelitis (EAE), another autoimmune T_H1 mediated disease that mimics human multiple sclerosis, anaphylactic shock can occur when the mice are challenged with certain myelin self peptides that initially were administered with adjuvant to induce the disease.

Results: Here we show that NOD mice, that spontaneously develop T1DM, can develop fatal anaphylactic reactions upon challenge with preparations of immunodominant GAD65 self peptides after immunization with these peptides to modify the development of T1DM.

Conclusions: These findings document severe anaphylaxis to self peptide preparations used in an attempt to devise immunotherapy for a spontaneous autoimmune disease. Taken together with the findings in EAE, these results suggest that peptide therapies designed to induce a T_H1 to T_H2 shift carry a risk for the development of anaphylactic reactivity to the therapeutic peptides.

Background

Type 1 diabetes mellitus (T1DM) is a T cell-mediated autoimmune disease characterized by lymphocytic infiltration of the pancreatic islets of Langerhans with subsequent destruction of the insulin-producing beta cells [1]. Non-obese diabetic (NOD) female mice, a murine model for T1DM, spontaneously develop diabetes by 30

weeks-of-age, with infiltrating cells appearing around the pancreatic islets as early as at 3–4 weeks-of-age [2].

T1DM susceptibility in the NOD mouse is linked to I-A^{g7}, the murine MHC class II gene that encodes a histidine at position 56 and a serine at position 57 in the β chain, in place of the more frequent proline 56β and aspartic acid 57β [3]. The development of diabetes is prevented in NOD.PD mice (which are NOD mice with I-A^{g7}) that carry a β chain transgene with site-specific mutations that restore proline and aspartic acid at positions 56β and 57β, respectively [4]. Furthermore, because of the two amino acid changes in the additional (transgenic) MHC class II allele β chain in NOD.PD mice, NOD.PD mice recognize three additional peptide epitopes in the glutamic acid decarboxylase 65 (GAD65) autoantigen [5].

Among beta-cell autoantigens, GAD65 is an important initial target of the immune response that results in beta-cell destruction and diabetes, in both humans and NOD mice [6–9]. While both humoral and cellular responses to GAD65 occur as early as 4 weeks of age in NOD mice [8], there is considerable evidence that beta-cell-specific T_H1 cells are the effectors of T1DM, whereas T_H2 cells appear to have a protective role [10]. Accordingly, a shift of the autoimmune response from T_H1 to T_H2 predominance has represented a promising strategy for prevention of diabetes and other T_H1-mediated autoimmune diseases.

For example, administration of GAD65 to young NOD mice has been shown to prevent insulitis and diabetes [8,9], apparently via induction of CD4+ regulatory T cells with a T_H2 phenotype [10]. Similarly, treatment with immunodominant peptides of myelin can prevent or reverse experimental autoimmune encephalomyelitis (EAE), a T_H1-associated inducible "autoimmune" disorder that is widely used as a model for human multiple sclerosis [11–13].

Unfortunately, recent work indicates that the application of strategies to shift autoimmune responses from T_H1 to T_H2 predominance is not without risk. Thus, some of us recently showed that administration of two self peptides that can induce EAE, myelin proteolipid protein peptide 139 to 151 (PLP139-151) or myelin oligodendrocyte glycoprotein peptide 35–55 (MOG35-55), can result in severe anaphylactic reactions [14]. This result clearly indicated that severe allergic reactions to self peptides can occur in mice that have been **induced** to express pathology (i.e., EAE) related to "autoimmunity" to these peptides. However, it was initially unclear whether anaphylactic reactivity also could be elicited to self peptides that have been implicated in the development of a **spontaneous** autoimmune disorder.

In the present study, we show that anti-peptide autoantibodies and fatal anaphylactic reactions can be elicited by immunodominant GAD65 peptides in NOD mice that have been injected with these peptides intraperitoneally in incomplete Freund's adjuvant (IFA), as part of an attempt to induce "tolerance" and prevent the spontaneous development of T1DM. Moreover, while this manuscript was in review, Liu and colleagues reported that anti-peptide autoantibodies and fatal anaphylaxis can be induced in NOD mice that have been immunized with insulin B chain peptides B:9–23 or B:13–23 [15]. However, in the Liu *et al.* study, the peptides were administered subcutaneously in saline without adjuvant. As reviewed in Liu *et al.*, [15] several lines of evidence indicate that amino acids 9–23 of the insulin B chain also represent a major target of anti-islet autoimmunity in T1DM. Taken together with the findings reported herein, this work indicates that anaphylactic reactions can be elicited in mice that have been immunized with pancreatic islet-associated self-peptides that also represent significant targets of autoimmunity in T1DM.

Results

Anaphylactic responses to GAD65 and PD peptides

In an attempt to induce a T_H2 shift, [19,20] 8 to 9 week old female NOD mice (I-A^{g7}) were immunized by 3 weekly i.p. injections of the immunodominant G7 peptides (GAD 206–226/217–236/286–300) or of the additional GAD65 peptides identified in NOD.PD mice (I-$A^{NOD/PD}$) (GAD 333–345/K458-470R) in IFA [5]. As noted in the background section, PD peptides are not immunodominant in NOD mice. Indeed, we originally included the PD-immunized group because PD peptides are the immunodominant epitopes that are presented in transgenic NOD.PD mice that do not get diabetes [4,5]. Because of their unknown, and potentially even protective, role in the diabetes-resistant NOD.PD strain, we felt that it was important to assess whether, through a peptide therapy regimen, PD peptides might be able to protect against diabetes by shifting T_H1 to T_H2 responses in NOD mice. As our study unfolded, and we found that G7 peptide therapy induced anaphylactic reactivity in NOD mice, we felt that it was important to evaluate whether PD peptides might also induce allergic responses in the NOD strain.

As demonstrated in our study, immunization of NOD mice with PD peptides can induce both a specific IgG1 response and also anaphylactic reactivity. On the other hand, as might be predicted, PD peptides induced a less robust IgG1 response (Figure 2) and also a lower incidence and severity of anaphylaxis (see Table 1 and Figure 1) when injected into NOD mice than did G7 peptides. In an attempt to induce anaphylactic reactivity to peptides known to induce T_H2 responses associated with allergic reactions, NOD mice were immunized using the same

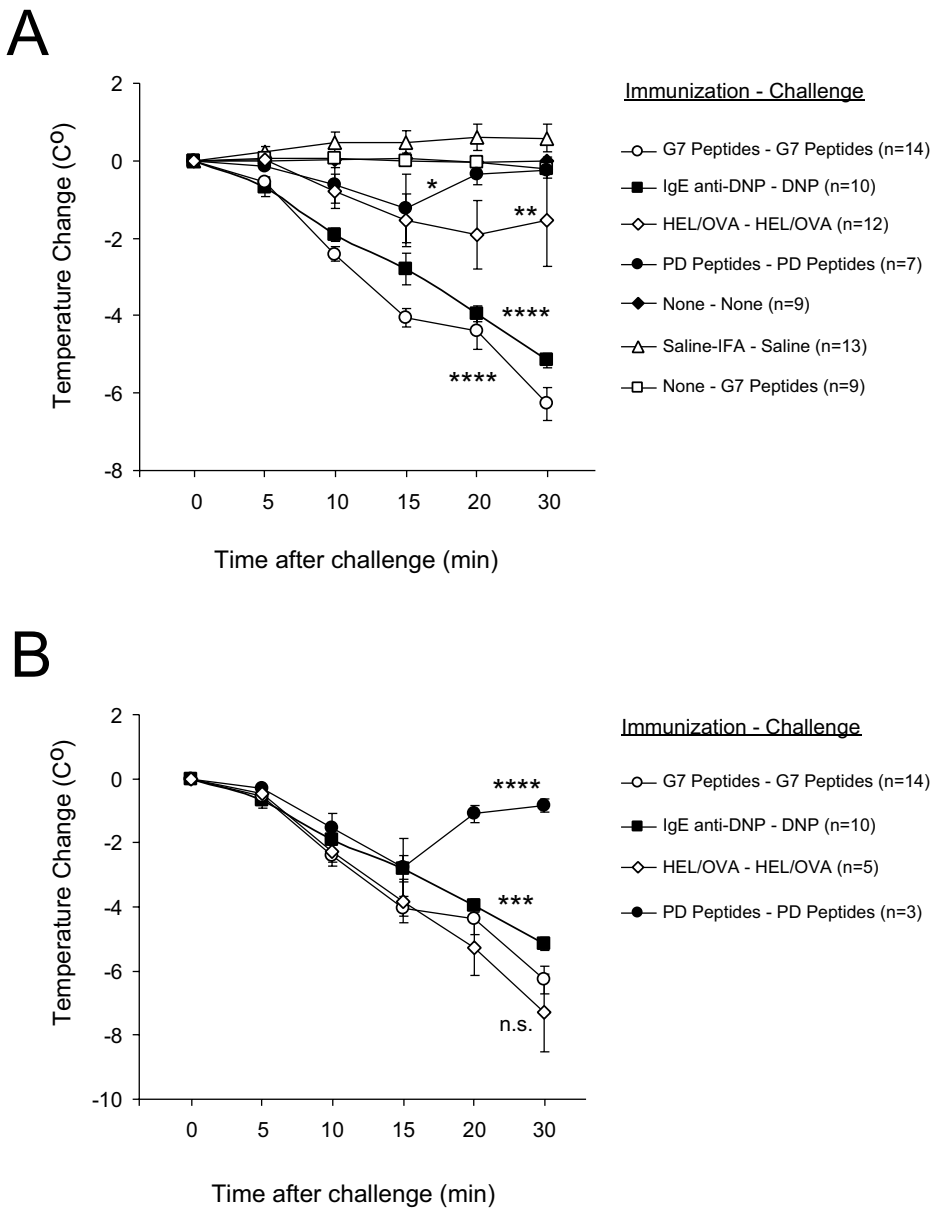

Figure 1

Challenge with G7 or PD GAD65 peptides caused anaphylactic shock. NOD mice were not injected ("none") or received 3 weekly i.p. injections with saline (as a control) or the indicated peptide preparations in IFA, and, except for the None/None group, were challenged with saline or peptides in saline i.p. 4 weeks after the last immunization/injection (see Methods). IgE-mediated passive systemic anaphylaxis was induced in mice that had been sensitized with an i.p. injection of anti-DNP IgE and then challenged i.v. 24 h later with DNP-HSA. Body temperature was measured at 5 minute intervals after challenge for 30 minutes or until death. Data are shown as mean +/- s. e. m. (A) Data for all mice, including those that gave no detectable anaphylactic response (i.e., temperature change of <1C° and no clinical signs of anaphylaxis). *, **, **** $P < 0.05$, 0.01, or 0.0001 vs any negative control group (i.e., None/None, Saline-IFA/Saline, None/G7 peptides) by ANOVA. (B) Data for mice that exhibited anaphylaxis. ***, ****, n.s., $P < 0.001$, 0.0001, or not significant ($P > 0.05$) vs G7 Peptides/G7 Peptides.

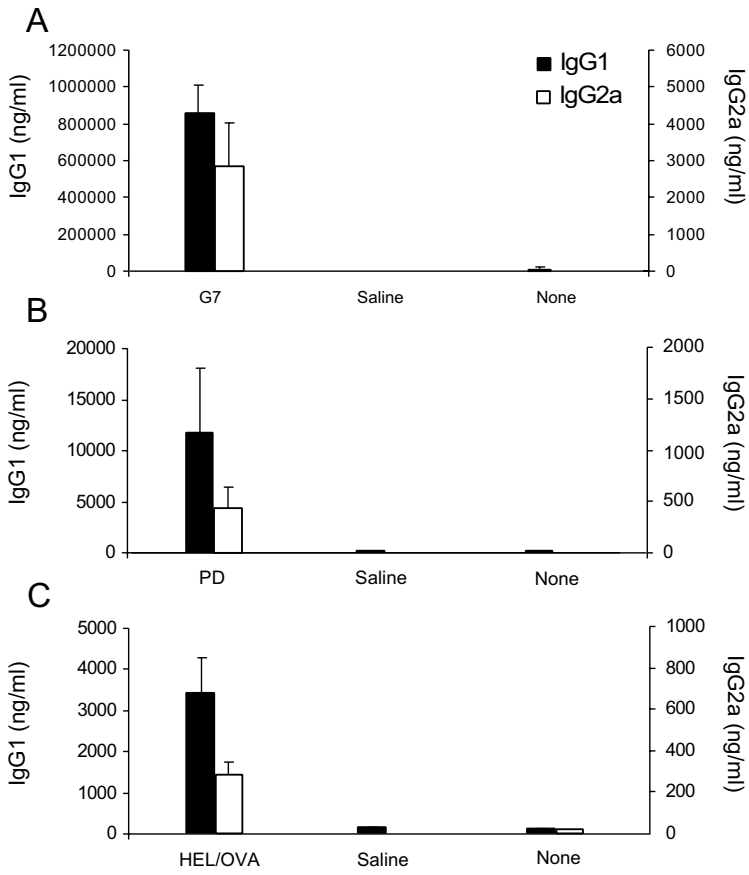

Figure 2

Peptide specific IgG1 and IgG2a antibodies in NOD mice immunized with G7 (A), PD (B), or HEL/OVA (C) peptides. Serum was collected 2–3 days before challenge with G7, PD, HEL/OVA or saline. IgG1 and IgG2a antibody responses specific for the G7, PD or HEL/OVA peptide epitopes were analyzed by ELISA. Each mouse was tested individually at a serum dilution of 1:20,000 for IgG1, and 1:25 for IgG2a for mice immunized against G7 peptides and 1:500 for IgG1 and 1:200 for IgG2a for mice immunized against PD or HEL/OVA peptides. Data are shown as mean +/- s. e. m. "Saline" = mice injected with saline/IFA; none = non-injected naïve mice.

Table 1:

Immunization[a]	Challenge[b]	Number of Mice with Anaphylaxis		
		Temperature Decrease ≥ 1 °C	Death (all mice)	Death (in mice with anaphylaxis)
G7 peptides	G7 peptides	14/14 (100%) *	12/14 (86%)**	12/14 (86%)***
PD peptides	PD peptides	3/7 (43%) †	1/7 (14%)	1/3 (33%)
HEL/OVA	HEL/OVA	5/12 (42%) ‡	4/12 (33%) ‡‡	4/5 (80%) ‡‡‡
Saline	Saline	0/13 (0%)	0/13 (0%)	-
None (no injection)	None (no injection)	0/9 (0%)	0/9 (0%)	-
No injection	G7 peptides	0/9 (0%)	0/9 (0%)	-
Passive Systemic IgE-induced Anaphylaxis				
IgE anti-DNP	DNP-HSA	10/10 (100%)#	0/10 (0%)	0/10 (0%)

a Immunizations in IFA **b** Challenge in 0.9% NaCl (saline) * P = 0.0058 vs. PD challenged, P = 0.0012 vs. HEL/OVA challenged, P < 0.0001 vs. G7 challenged non-immunized mice, saline challenged or "none" groups; † P = 0.031 vs saline challenged; ‡ P < 0.05 vs G7 challenged non-immunized mice, saline challenged or "none" groups; # P = 0.0147 vs PD challenged, P = 0.0053 vs HEL/OVA, P < 0.0001 vs G7 challenged non-immunized mice, saline challenged or "none" groups.; ** P = 0.0138 vs. HEL/OVA challenged, P < 0.0001 vs DNP-HSA challenged; ‡‡ P = 0.048 vs saline challenged; *** P < 0.0001 vs DNP-HSA challenged; ‡‡‡ P = 0.0037 vs DNP-HSA challenged; all other comparisons are P > 0.05 (Fisher exact test).

protocol with hen egg lysozyme and ovalbumin peptides (HEL 81–96, OVA 323–339) [21–23]. As a negative control, NOD mice received 3 weekly injections of saline emulsified in IFA. Four weeks after the last of the 3 i.p. injections of peptides/IFA or saline/IFA, mice injected with peptides/IFA were challenged i.p. with the same peptides used for the immunizations dissolved in saline, whereas mice that had been injected with saline/IFA were challenged with saline alone. By the day of challenge, 10–15% of all mice had developed diabetes, with the exception of the mice in the saline group (0%).

All of the mice challenged with G7 peptides developed severe anaphylactic shock (100%; 14/14), with the majority of them dying within 30 minutes after the injection (86%; 12/14) (Table 1). In addition to the classical signs of anaphylaxis, such as reddening of the skin, prostration and respiratory impairment, the mice underwent a dramatic drop in body temperature (Fig. 1), which confirmed the presence of anaphylactic shock. Moreover, the death rate from anaphylaxis was substantially higher than in any other group in which anaphylaxis occurred (Table 1).

On the other hand, the clinical and physiological features of anaphylaxis elicited by the G7 peptides were similar to those observed in age- and gender-matched NOD mice undergoing IgE-mediated passive systemic anaphylaxis (Fig. 1). Although the death rate was significantly higher in the G7 challenged NOD group (86%; 12/14) compared to the IgE-sensitized, DNP-HSA challenged group (none) (P = < 0.0001 by Fisher's exact test, Table 1), those mice in either group that developed anaphylaxis exhibited quite similar drops in body temperature (Fig. 1B). Similarly, while the group of mice that was challenged with G7 peptides exhibited a higher incidence of anaphylactic responses than did the group challenged with HEL/OVA peptides (Table 1), the temperature changes (Fig. 1B) and death rates (Table 1) in mice that did develop a reaction were quite similar.

None of the naïve age/gender-matched NOD mice (these mice received no injection prior to challenge) that were challenged with G7 peptides showed any signs of anaphylaxis (0/9; P < 0.0001 by Fisher's exact test for comparison vs. G7 immunized, G7 challenged mice) (Table 1, Fig. 1). This result indicates that priming of these mice with G7 peptides is required for the elicitation of the allergic response.

Of the mice immunized with the PD peptides, that are not immunodominant in NOD mice, 43% (3/7) developed anaphylactic shock at the time of challenge with PD peptides (Table 1). Thus, the incidence of anaphylactic shock in mice immunized and challenged with PD peptides was significantly lower than that in mice immunized and challenged with immunodominant G7 peptides (P = 0.0058 by Fisher's exact test). Moreover, of those PD-immunized, PD-challenged mice that did exhibit an anaphylactic reaction, the drop in temperature was less sustained than that in those mice in the other groups that exhibited anaphylaxis (Fig. 1B) and only 1 of these mice died (33%) (Table 1). In accord with these results, immunization of the NOD mice with PD peptides produced a less robust specific IgG1 antibody response than did immunization with the immunodominant G7 peptides (see below). As expected, none of the mice immunized with saline/IFA

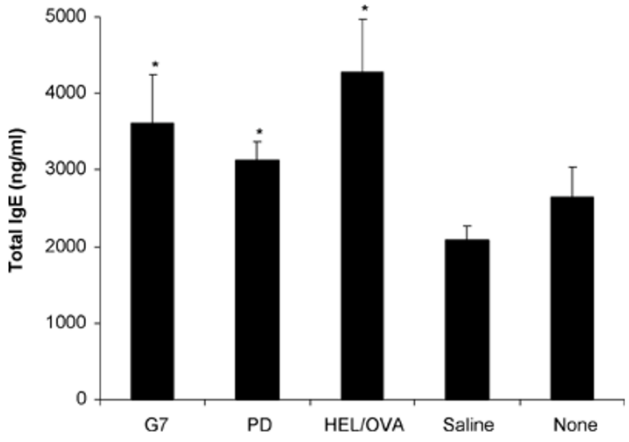

Figure 3
Immunization with G7, PD, or HEL/OVA peptides induced modest changes in total IgE. Total IgE serum concentrations were measured at a dilution of 1:100 by sandwich ELISA. Data are shown as mean +/- s. e. m. * = $P < 0.05$ vs. levels in saline-treated mice.

alone developed anaphylaxis upon i.p. challenge with saline (Table 1, Fig. 1).

Notably, in the mice immunized with G7, PD or HEL/OVA emulsified in IFA, anaphylactic responses were also provoked by the third i.p. immunization with peptides (10/12 in the G7 group; 3/4 for PD, 3/10 for HEL/OVA). However, these anaphylactic responses were less severe than those induced by subsequent peptide challenge of the same mice, with a less dramatic drop in body temperature (data not shown) and no deaths. Finally, although the numbers of mice that had developed diabetes by the day of peptide challenge in each of the immunized groups was small (10–15%), there were no statistically significant differences in the incidence of anaphylactic reactions in these mice vs. mice that were normoglycemic at the time of peptide challenge.

IgG1, IgG2a and IgE responses

Antibody responses were analyzed by ELISA in serum obtained 2 to 3 days before the 4 week challenge with peptides or saline. Mice immunized with the G7 peptides had high IgG titers against the G7 peptides, with levels of IgG1 being significantly higher than those of IgG2a ($P < 0.0001$ by Mann-Whitney U test, 2-tailed; Figure 2A). Anti-G7 IgG antibodies generally were not detectable in the other groups (PD- or HEL/OVA- immunized, or IFA alone) or in the non-injected (naïve) mice. The two exceptions were a single non-immunized mouse with anti-G7 IgG1 antibodies (at a serum concentration of 90 μg/ml), and one mouse immunized with HEL/OVA with anti-G7 IgG2a antibodies (at a serum concentration of 492 ng/ml). Anti-peptide IgG1 and IgG2a antibodies also were detected in PD- (Figure 2B) and HEL/OVA- (Figure 2C) immunized mice, although the magnitude of the antibody responses to these peptides (especially the IgG1 response) were substantially less than those to the G7 peptides.

Total IgE concentrations were slightly, but significantly, higher in the peptide-immunized groups (G7, PD, or HEL/OVA) compared to those in mice injected with IFA and saline alone (Figure 3). However, the serum concentrations of total IgE were very similar in the mice that had been immunized with G7, PD or HEL/OVA peptides (Figure 3).

Discussion

This study clearly demonstrates that i.p. immunization of NOD mice with preparations of GAD65 self peptides in IFA can cause a marked shift towards a T_H2 like response, as reflected by high levels of IgG1. Similarly, Liu *et al.* recently demonstrated that strong IgG1 responses can be induced in NOD mice that have been immunized subcutaneously with insulin B chain peptides administered in physiological saline [15]. However, both studies showed that anaphylaxis can be induced in such mice upon subsequent re-challenge with preparations of the peptides used for immunization [15]. Moreover, the anaphylactic reactions in mice that had been immunized and challenged with G7 peptides were severe, with reductions in body temperature that were very similar to those observed in mice exhibiting IgE-dependent passive systemic anaphylaxis and with a very high fatality rate (12/14 mice, or 86%). Anaphylaxis also developed in some NOD mice that had been immunized and challenged with preparations of PD peptides (that are not immunodominant in NOD mice), although both the drop in body temperature and the death rate in these mice were significantly less than those observed in the mice immunized and challenged with G7 peptides.

There were both similarities and differences between our findings in the NOD mouse model of T1DM and those in the EAE model [14]. Expression of EAE requires specific immunization with self peptides (e.g., PLP 139–151 or MOG35-55), and these peptides generally are administered in complete Freund's adjuvant (CFA). By contrast, T1DM develops spontaneously in NOD mice. On the other hand, induction of anaphylactic reactivity in NOD mice appeared to require immunization of the mice with GAD65 peptides (in this model, in IFA), as naïve NOD mice challenged with G7 peptides exhibited no detectible reactions, and none of them died (Table 1). Thus, in both the EAE model [14] and the NOD T1DM model (this study, and that of Liu *et al.*, [15]) some form of artificial

"immunization" with a self peptide preparation appears to be required for the development of anaphylactic reactivity to "self". This of course is not a surprising result. Indeed, it is challenging to conceive of any possible selective advantage that would be conferred by a propensity to develop, under "natural" conditions, potentially fatal allergic reactions to components of self. It remains to be determined whether self peptide immunization protocols that induce anaphylactic reactivity do so simply because of the manner in which they present large amounts of self peptides to the immune system, or because of other factors, such as the presence in the peptide preparations of aggregates or other components beside self peptide monomers.

Whatever the underlying reason(s) for the development of anaphylactic reactivity to these self peptide preparations, in both the EAE and the NOD T1DM models, anaphylactic reactions occurred in mice that had developed strong IgG1 responses to the relevant self peptides, with only modest changes in total IgE levels. In humans, antigen-specific anaphylactic reactivity is thought to be mediated solely (or primarily) by IgE antibodies, whereas it has long been known that either IgE or IgG1 antibodies can mediate anaphylaxis in mice (reviewed in [14,15] and [17]). However, it has been reported that IgG1-dependent anaphylaxis in the mouse is associated with substantially less histological evidence of mast cell degranulation than is observed in IgE-dependent anaphylaxis in that species [17]. In neither of the models of "autoimmunity" that we have studied (i.e., EAE, T1DM in NOD mice) was anaphylaxis associated with histological evidence of substantial mast cell degranulation [14] (data not shown). Taken together, these findings suggest that IgG1 antibodies contribute importantly to the development of anaphylaxis in both of these models. On the other hand, we can not rule out some role for IgE antibodies in these reactions.

Indeed, Liu *et al*. [15] found that, in NOD mice that had been immunized with peptide B:9–23, treatment with both anti FcγRII/RIII and anti-IgE monoclonal antibodies was required to prevent anaphylaxis upon challenge with the peptide. Interestingly, however, Liu *et al*. [15] did not detect IgE antibodies to B:9–23 or B:13–23 in the serum of their NOD mice. By contrast, mice that had been immunized with B:9–23 peptide at 10 or 100 μg/dose exhibited a robust and dose-dependent IgG1 antibody response to the peptide [15]. Thus, in both B:9–23 peptide-associated anaphylaxis (Liu *et al*. [15]) and GAD65 peptide-associated anaphylaxis (our study), anti-peptide IgG1 antibodies contribute to the response. However, IgE antibodies also appear to contribute to anaphylaxis to B:9–23 peptides [15], and may also be involved in our model.

One point not yet clarified by the comparison of the present results, those of Liu *et al*. [15], and those of Pedotti *et al*.[14] is whether the influence of thymic expression of the self peptide on the propensity to develop anaphylactic reactivity differs in the EAE and NOD T1DM models. In the study by Pedotti et al. [14], it was noted that the two self peptides that induced anaphylactic reactivity, MOG 35–55 and PLP139–151, are not expressed in the thymus, whereas the two peptides tested that did not induce anaphylactic reactivity, PLP_p 178–191 and $MBPA_C$1–11, are expressed at that site. However, both GAD65 and GAD67 mRNA can be detected in the thymic medullary epithelial cells in mice [24]. Thus, despite thymic expression of GAD65 and GAD67 at the level of mRNA, NOD mice spontaneously develop autoreactivity to these islet (and brain) expressed proteins, and re-challenge of mice that have been immunized with peptides from GAD65 results in severe anaphylactic reactions. On the other hand, expression of GAD65 or GAD67 protein in the thymus has not yet been reported. Similarly, as reviewed in Liu *et al*. [15], although several lines of evidence indicate that insulin is present in the thymus of mice and humans, it is possible that the specific insulin peptides that induced anaphylaxis in their study are not ordinarily present in that site. As a result, it has not yet been demonstrated that anaphylactic reactions can develop to self peptides that are expressed in the thymus.

It should be emphasized that NOD mice have a partial defect in thymic negative selection [25,26], a defect in FcγRI-IB (that can negatively regulate anaphylactic reactions [27,28]), and perhaps other genetic polymorphisms that may result in immunological hyperresponsiveness. The same is likely to be true in at least some patients with type 1 diabetes, and in patients in the pre-diabetic phase. Therefore, because of the risk of induction of anaphylactic sensitization, extreme caution needs to be used in developing any type of antigen-specific immunosuppressive therapy for the prevention or treatment of T1DM. This caution probably should be extended to all attempts to shift immune responses to self or foreign antigens from a T_H1 to a T_H2 response. Indeed, in a recent phase II clinical trial, 9% of MS patients given an altered peptide ligand (APL) of a myelin basic protein epitope developed immediate hypersensitivity reactions after multiple injections of the APL [29]. Thus, it would appear that great care must be taken when injecting preparations of putative "tolerogens" in attempts to suppress T_H1-mediated autoimmune diseases.

Methods

Mice

Female NOD/LtJ mice (The Jackson Laboratory, Bar Harbor, ME), were maintained on Lab Diet 5K52 (Purina, St Louis, MO), under filter-top barrier conditions. Mice were

tested three times a week for glycosuria using Chemstrip uGK (Roche Diagnostics, Indianapolis, IN), and considered diabetic when tested positive (glucose levels above 100 mg/dL), on three consecutive occasions.

Peptides

Three peptide pools consisted of: G7 (GAD 206–226, GAD 217–236, GAD 286–300), PD (GAD K458-470R, GAD 333–345), and hen egg lysozyme/ovalbumin (HEL/OVA; HEL 81–96, OVA 323–339). All peptides were synthesized by Research Genetics (Huntsville, AL) and were confirmed > 90% pure by HPLC and Mass Spectrometry analysis.

Immunizations

Mice (8–9 weeks old) received three weekly intraperitoneal (i.p.) injections of 100 μl containing a mixture of 200 μg each of the G7 peptides, the PD peptides or the HEL/OVA peptides, dissolved in 50 μl of sterile, pyrogen-free 0.9% NaCl ("saline") and emulsified in an equal volume of incomplete Freund's adjuvant (IFA) (Difco Laboratories, Detroit, MI). A peripheral blood sample was obtained 2 to 3 days before challenge and was analyzed for antibody response by ELISA. Mice were challenged four weeks after the third immunization (at 15-16 weeks-of-age) by i.p. injection of the same peptide pools (200 μg of each peptide) dissolved in saline. Mice were observed for 30 minutes after challenge for signs of anaphylaxis, and temperature was taken at intervals of 5 minutes. As negative control groups, mice were immunized with an emulsion of IFA and saline and challenged with saline, and age-gender-matched non-immunized mice were challenged with the G7 mixture (containing 200 μg of each peptide in pool) dissolved in 50 μl of saline. As an additional control, temperature measurements were taken from unmanipulated (non-injected) naïve mice.

Passive systemic anaphylaxis

For passive systemic anaphylaxis, 15-week-old NOD mice were injected i.p. with 20 μg anti-DNP-IgE (IgE hybridoma = H1 DNP-ε-26) [16] dissolved in 200 μl HMEM (Gibco-BRL, Gaithersburg, MD) with PIPES buffer (0.47 g/l, Sigma, St. Louis, MO). Twenty-four hours later, mice were challenged intravenously (i.v.) with 200 μg DNP-HSA (Sigma) dissolved in 200 μl saline [17].

Temperature measurement

Rectal temperatures were taken using Physitemp (Clifton, NJ). Basal temperatures were recorded before challenge, and temperature readings were taken at 5 minute intervals until death from anaphylaxis or 30 minutes post injection, whichever occurred first. Temperature measurements were performed in a "blinded" fashion.

IgG1 and IgG2a antibody measurements

G7, PD and HEL/OVA peptide-specific IgG1 and IgG2a responses were measured in duplicate with mouse sera collected 1 to 3 days before challenge. EIA/RIA 96-well plates (Corning Incorporated, Acton, MA) were coated overnight at 4°C with a 100 μl mixture of each peptide preparation in a pool for a total peptide concentration of 30 μg/ml diluted in physiologic saline. After 3 washes with phosphate-buffered saline (PBS) and 0.05% Tween 20 (Sigma), plates were blocked with PBS plus 2% BSA (Sigma), and 0.02% sodium azide (Sigma), for 2 hours at room temperature (RT). Serum samples were diluted in blocking buffer and incubated for two hours at RT. After 1 hr incubation at RT with 50 μl/well of biotinilated secondary antibodies, plates were developed with Eu-labelled Streptavidin (PerkinElmer Life Sciences, Boston, MA) followed by Enhancement solution (PerkinElmer Life Sciences) and read in a 1234 Delfia Fluorometer (PerkinElmer Life Sciences). Serum Ig values were interpolated from standard curves obtained by coating the plates directly with purified IgG1 or IgG2a (PharMingen) at a starting concentration of 500 ng/ml, according to the manufacturer's instructions.

Total IgE antibody measurement

Total IgE was measured in duplicate with mouse serum at 1:100 dilution by sandwich ELISA (PharMingen) according to the manufacturer's instructions [18].

Authors' Contributions

Rosetta Pedotti and Maija Sanna participated in the design of the experiments and performed the peptide immunizations and challenges in the mice, measurements of anaphylactic responses and ELISA immunoassays for antibodies. Rosetta Pedotti and Maija Sanna contributed equally to this study, including collaborating in writing the first draft of the manuscript. Mindy Tsai participated in the design and execution of the study and the drafting of the manuscript. Jason DeVoss performed some of the ELISA immunoassays for antibodies. Lawrence Steinman, Hugh McDevitt, and Stephen J. Galli participated in experimental design, interpretation of the results, and revision of the manuscript. All authors read and approved the final version of the manuscript.

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