

Preventing peptide-induced anaphylaxis: Addition of C-terminal amino acids to produce a neutral isoelectric point

Edwin Liu, MD,^{a,c} Hiroaki Moriyama, MD,^c Norio Abiru, MD,^c Johanna Paronen, MD, PhD,^c Devasenan Devendra, MD,^c Fred D. Finkelman, MD,^b and George S. Eisenbarth, MD, PhD^c

Denver, Colo, and Cincinnati, Ohio

Background: Progress in peptide immunotherapy for the treatment of autoimmune diseases has been hampered by reports of anaphylactic reactions in both mice and human subjects. Fatal anaphylaxis in nonobese diabetic (NOD) mice has been described after repeated subcutaneous insulin peptide B:9-23 immunizations. On the basis of observations that rapid systemic delivery of peptide to a sensitized mouse (eg, intravenous delivery) increases the anaphylactic response, it was hypothesized that slowing down the absorption of the peptide would prevent anaphylaxis.

Objectives: We sought to prevent anaphylaxis from B:9-23 peptide by altering the isoelectric point (pI) to neutral, thereby decreasing solubility and rate of absorption after subcutaneous injection.

Methods: B:9-23 peptide was modified by the addition of 2 arginine (RR) amino acids to the C-terminus to create B:9-23RR, thereby increasing the pI from 5.4 to 7.0. Both native and modified B:9-23 peptide were tested for the ability to induce anaphylaxis in a NOD mouse model of self-peptide anaphylaxis.

Results: This modification resulted in a peptide vaccine with decreased solubility when administered subcutaneously at a neutral pH. B:9-23RR significantly protected NOD mice from peptide-induced anaphylaxis compared with B:9-23 peptide.

Furthermore, B:9-23RR peptide retains its ability to induce insulin autoantibodies and prevent diabetes in NOD mice.

Conclusion: The modification of the pI of a peptide vaccine might be a generalizable method to prevent anaphylaxis without changing the immunologic properties. (*J Allergy Clin Immunol* 2004;114:607-13.)

Key words: Anaphylaxis, diabetes, insulin, peptide, immunotherapy, isoelectric point, B:9-23, NOD, prevention, vaccine, immunization, allergy

Identification of disease-related peptide antigens involved in autoimmune and hypersensitivity disorders have led to studies of peptide vaccination for the prevention or treatment of these disorders. Vaccine trials in the nonobese diabetic (NOD) mouse with self-peptides, such as insulin B:9-23 and glutamic acid decarboxylase,^{1,2} were promising in that they suppressed the development of diabetes. Peptide therapy was compromised, however, by the development of anaphylaxis in these mice.^{3,4} Anaphylactic reactions have been reported in studies in which mice were immunized with proteolipid protein (PLP)-derived or myelin-oligodendrocyte-glycoprotein-derived peptides in an attempt to suppress the development of experimental autoimmune encephalitis, a mouse model of multiple sclerosis.⁵ Furthermore, hypersensitivity reactions have caused the premature discontinuation of PLP-derived peptide therapy in human subjects with multiple sclerosis,^{6,7} and hypersensitivity reactions have occurred in human trials of cat allergen peptides as a therapy for cat allergy.⁸

Studies of anaphylaxis in the mouse have established roles for IgG and IgE, histamine, and platelet-activating factor (PAF) in this disorder.^{3,9} In human subjects IgE and histamine are involved in anaphylaxis, and there is no evidence to date to support the role of IgG or PAF. Although a critical amount of mast cell and basophil surface cross-linking of IgE is necessary to cause cell activation and histamine release, each cell might have different thresholds for activation, such that there might be various levels of reaction to a sensitized antigen, ranging from subclinical responses to fulminant anaphylactic shock. An approach to safer peptide vaccination would be to design peptides for vaccination that reduce the risk of anaphylaxis. However, such peptides would have to retain

From ^athe Section of Gastroenterology, Hepatology and Nutrition and The Children's Hospital, Denver; ^bthe Division of Immunology, University of Cincinnati College of Medicine, Cincinnati Veterans Administration Medical Center, and Children's Hospital Medical Center, Cincinnati; and ^cthe Barbara Davis Center for Childhood Diabetes and the Department of Pediatrics, University of Colorado Health Sciences Center, Denver.

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Reprint requests: Edwin Liu, MD, Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, 4200 East 9th Ave, B140, Denver, CO 80262. E-mail: Edwin.liu@uchsc.edu.

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Abbreviations used

NOD: Nonobese diabetic
PAF: Platelet-activating factor
pI: Isoelectric point
PLP: Proteolipid protein

the original immunologic properties (eg, induction of a protective immune response) for which they were selected.

Our mouse model of anaphylaxis, which uses insulin peptide B:9-23 administered in saline, allows us to study the effect of peptide immunizations without adjuvant effects.³ Consistent with the described pathways of anaphylaxis in mice by Strait et al,⁹ B:9-23 peptide-induced anaphylaxis is mediated by both IgE and IgG through the actions of both histamine and PAF.³ Mice that received weekly injections of B:9-23 peptide subcutaneously in saline became sensitized to the peptide, as demonstrated by the development of high titers of IgG1 anti-B:9-23 in immunized mice. Consistently, after 6 weeks of repeated peptide injections, sensitized mice could be induced to anaphylaxis with additional injections of the B:9-23 peptide. Some sensitized mice failed to have anaphylaxis induction after subcutaneous B:9-23 peptide injection; however, intravenous injection of the same mice with the same peptide evoked a vigorous anaphylactic response that was invariably fatal. This suggested that rapid delivery of antigen into the systemic circulation of sensitized animals was important for the induction of anaphylaxis. In patients who experience an infusion reaction to a drug, decreasing the rate of infusion often improves tolerance to the medication.¹⁰ Thus decreasing the rate of antigen absorption into the systemic circulation might be able to prevent anaphylaxis, for example, by resulting in insufficient mast cell and basophil stimulation. On the other hand, slowing the rate of absorption might also serve to decrease the amount of medication absorbed per unit of time such that desensitization occurs.

On the basis of the hypothesis that slowing systemic absorption of the B:9-23 peptide would prevent anaphylaxis in sensitized mice, we modified B:9-23 to decrease its solubility in subcutaneous tissue and presumably prolong the absorption process by neutralizing the peptide's isoelectric point (pI). We hypothesized that altering a peptide's pI to neutral would prevent anaphylaxis. This was accomplished with B:9-23 peptide by adding 2 arginine residues (RR) to its C-terminus (thus creating B:9-23RR), which increased the pI from 5.4 (for native B:9-23) to 7.0 (for B:9-23RR) and decreased peptide solubility at neutral pH (eg, the physiologic pH of human tissue, 7.0-7.4).

In this study we demonstrate that use of the B:9-23RR peptide instead of B:9-23 avoids anaphylaxis in sensitized mice but preserves B:9-23 immunogenicity. This method of altering the pI of a peptide to prevent anaphylaxis after subcutaneous injection might be generalizable to other forms of peptide vaccinations.

METHODS

Mice

Female NOD mice were purchased from Taconic Laboratories (Germantown, NY) and housed in the pathogen-free animal colony at the Barbara Davis Center for Childhood Diabetes. The usual incidence of diabetes for female mice in this population is 80% by 30 weeks of age. Starting at 12 weeks, blood glucose is monitored weekly with Freestyle glucometers (Therasense Inc, Alameda, Calif). Every diabetic mouse (defined as a blood glucose level of >250 mg/dL on 2 consecutive measurements) is killed. Serum for antibody measurement is obtained every 4 weeks.

Peptides

Peptides B:9-23 (SHLVEALYLVCGERG), B:9-23RR (SHLVEALYLVCGERGRRR), B:9-23RRRR (SHLVEALYLVCGERGRRRR), and TT:830-843 (QYIKANSKFQITE) were purchased from Synpep Corporation. HPLC purified greater than 90%, and confirmed with mass spectrometry. Peptides were dissolved in sterile hospital-grade saline solutions, and pH was adjusted with 1 mol/L NaOH or 1 mol/L HCl as needed. Peptide concentrations were confirmed by means of UV absorption on the basis of the extinction coefficient. pIs were calculated by using the ExPASy proteomics server of the Swiss Institute of Bioinformatics and the Java-based Molecular Biologist's Workbench (JaMBW). The calculated pIs of B:9-23 and its altered forms are shown in Fig 1.

Sensitization and induction of anaphylaxis

Mice are sensitized with B:9-23, B:9-23RR, or B:9-23RRRR, starting at 4 weeks of age, by injecting 100 µg of peptide per dose subcutaneously in the scruff on days 1 to 5, day 8, and weekly thereafter. At 10 weeks of age, mice have overt signs of anaphylaxis with further injections and are considered sensitized. Once sensitized, additional injections of peptide are given to induce anaphylaxis (Fig 2).

Monitoring anaphylaxis

As described previously, the clinical anaphylaxis score was used to monitor anaphylaxis on the basis of activity and level of alertness: a score of 0 represented normal behavior, 1 represented decreased spontaneous activity, 2 represented decreased activity with falling over and a loss of the self-righting reflex, and 3 was assigned to mice that became moribund with cool cyanotic extremities.³ A score of 0 to 3 was given to each mouse before peptide injection and every 5 minutes after injection for the next 30 minutes. In addition, core temperature was recorded with a rectal probe thermometer (4600 Precision Thermometer and 451 Probe; YSI Inc, Yellow Springs, Ohio). Any mouse that became moribund or experienced prolonged anaphylaxis beyond 30 minutes was killed. Mice that recovered from anaphylaxis were restudied 1 or more weeks later. The nature and timing was highly characteristic of systemic hypersensitivity (including tachypnea, piloerection, and a decrease in body temperature) and occurred within a few minutes of peptide injection, typically ending after 30 minutes.¹¹

Antibody measurement

Serum levels of anti-insulin autoantibodies were measured with a high-throughput fluid-phase radioimmunoassay, as described previously.¹² Antibodies to insulin are absorbed by intact insulin only and not by B:9-23 peptide. Anti-peptide IgG1 antibodies were measured at a dilution of 1:500 by using a Europium-based ELISA also described previously.³ The results are given in counts per minute; an index was calculated on the basis of positive and negative standards. The formula for the anti-B:9-23 index is as follows:

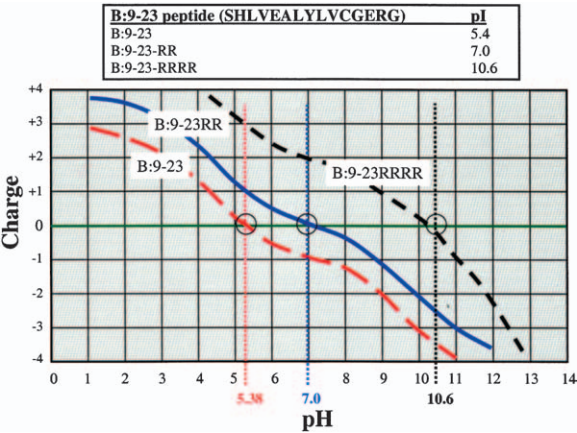


FIG 1. The pIs of B:9-23, B:9-23RR, and B:9-23RRRR are shown above. A peptide in solution in which pH equals pI has a zero charge, maximizing peptide aggregation and precipitation. Because the pH of the solution is further from the pI, a net charge develops, increasing peptide solubility.

(Sample cpm - Negative control cpm)/(Positive control cpm - Negative control cpm).

Anti-peptide antibodies in immunized mice were previously reported in dilutions of 1:30,000, but the lower limit of detection was at a dilution of 1:10⁶. Absorption experiments were performed by using the ELISA technique, as described above, but before the 1:500-diluted sera was added to the 96-well plate, sera were preincubated with no peptide, B:9-23RR, or whole insulin (1 mg/mL). After 20 minutes, sera were loaded onto the 96-well plate, and the remainder of the protocol was completed. To date, we have been unable to detect peptide-specific IgE antibodies, despite evidence of the role of IgE in this model system.

RESULTS

Peptide-sensitized mice that receive 100 µg of B:9-23 per dose almost always experience anaphylaxis after 11 or more injections (57/59 mice had anaphylaxis). However, peptide-sensitized mice rarely (2/45) have anaphylaxis when injected with 100 µg of B:9-23RR per dose, despite prior sensitization with the native or modified peptide (*P* < .0001, Fisher exact test). In contrast, intravenous injection of only 1 µg of B:9-23RR induced anaphylaxis in 2 of 3 sensitized mice, and intravenous injection of 10 µg killed 2 of 2 mice (Table I). Of note, 100-µg subcutaneous doses of peptide are used for sensitization because 10-µg doses do not sensitize mice for anaphylaxis (data not shown).

B:9-23 and B:9-23RR cross-react in inducing anaphylaxis, as shown in Table I. Mice sensitized with B:9-23RR will experience anaphylaxis to B:9-23 injected subcutaneously or intravenously. Likewise, mice sensitized with B:9-23 will have anaphylaxis to B:9-23RR injected intravenously (but rarely if injected subcutaneously). Thus B:9-23 and B:9-23RR are sufficiently similar to induce anaphylactic reactions, regardless of which peptide was used initially to sensitize mice. Consistent with this, ELISA data show that NOD mice immunized with B:9-23RR have anti-peptide antibodies that bind to B:9-

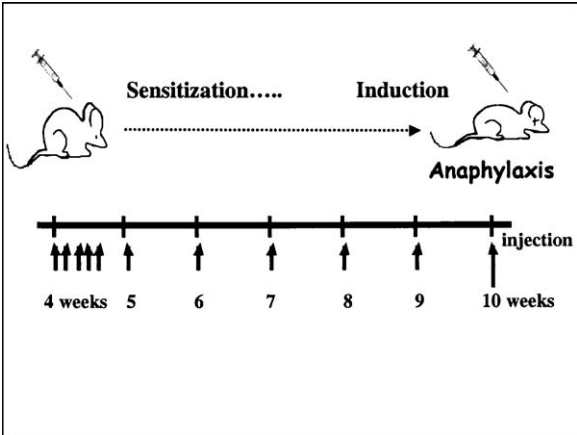


FIG 2. Protocol for sensitization and induction of anaphylaxis in mice with B:9-23 and related peptides. Starting at 4 weeks of age, mice are injected with peptide at 100 µg per dose on days 1 to 5, day 8, and then weekly thereafter. After 10 weeks of age, mice are considered to be sensitized, and further injections induce anaphylaxis.

TABLE I. Dose response to induce anaphylaxis with peptides B:9-23 and B:9-23RR given intravenously and subcutaneously in sensitized mice

		Anaphylaxis to sensitizing peptide (100 µg) SQ	
Induction dose (µg)	Route	B:9-23	B:9-23RR
B:9-23 induction			
1	IV	7/8	
10	IV	3/3	
100	IV	1/1	
10	SQ	1/6	
100	SQ	45/47	12/12
B:9-23RR induction			
1	IV	4/6	
10	IV	2/2	
100	IV	ND	
10	SQ	ND	
100	SQ	1/24	1/21

IV, Intravenous; SQ, subcutaneous; ND, not done. Peptides B:9-23 and B:9-23RR are cross-reactive, as evidenced by the ability of either peptide to induce anaphylaxis, regardless of which peptide was used to sensitize animals. Once sensitized, as little as 1 µg per dose intravenously of either peptide is required to induce anaphylaxis. The subcutaneous dose required to induce anaphylaxis for B:9-23 is 100 µg/dose. On the other hand, B:9-23RR is resistant to anaphylaxis at the same dose.

23-coated plates (Fig 3, A). Furthermore, antibodies from the sera of B:9-23-immunized mice are not absorbed by intact insulin but can be absorbed by B:9-23RR peptide (Fig 3, B).

B:9-23RR is believed to be less likely than B:9-23 to induce anaphylaxis when injected subcutaneously because of the former peptide’s lower solubility at neutral pH and slower entry into the systemic circulation. Evidence of pH-dependent solubility of B:9-23 and B:9-23RR is shown in Fig 4, in which the concentration of either peptide in solution is shown as a function of pH. B:9-23 is least soluble at pH 5 and nearly fully soluble at pH 7

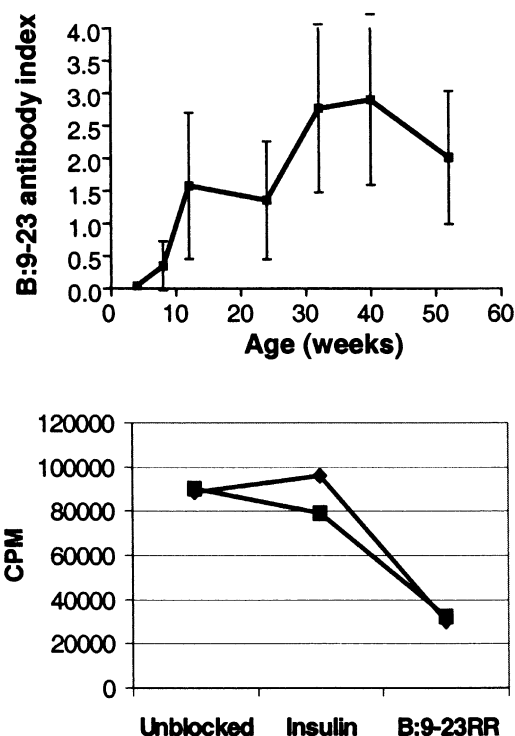


FIG 3. **A**, Antibodies to B:9-23 are induced in NOD mice on immunization with B:9-23RR, indicating strong cross-reactivity between the 2 peptides. **B**, Anti-B:9-23 antibodies from NOD mice immunized with B:9-23 are absorbed by B:9-23RR but not by whole insulin.

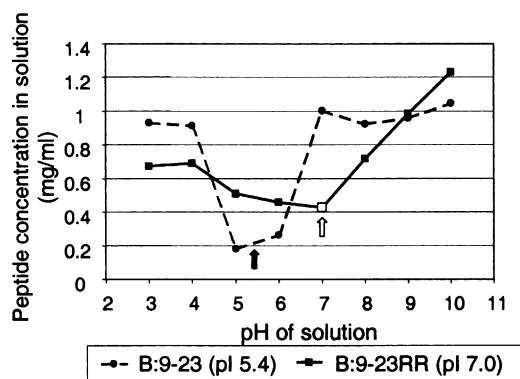


FIG 4. Solubility of B:9-23 and B:9-23RR as a function of pH. Concentrations of B:9-23 and B:9-23RR in solution vary according to the pH of the solution. Both peptides are at an initial concentration of 1 mg/mL. Peptide concentration in actual solution was measured by means of UV absorption after pH adjustment and removal of the precipitate by means of centrifugation. B:9-23 is maximally soluble at pH 7, whereas B:9-23RR is least soluble at this pH. The *closed arrow* indicates a pl of B:9-23, and the *open arrow* indicates a pl of B:9-23RR.

through 10, whereas B:9-23RR is least soluble at pH 7, with increased solubility at a pH of less than 5. Consistent with this, injection of 100 μ g of B:9-23RR at a pH of less than 5 induced anaphylaxis in 17 of 22 sensitized mice. Therefore it is believed that subcutaneous injection of B:9-

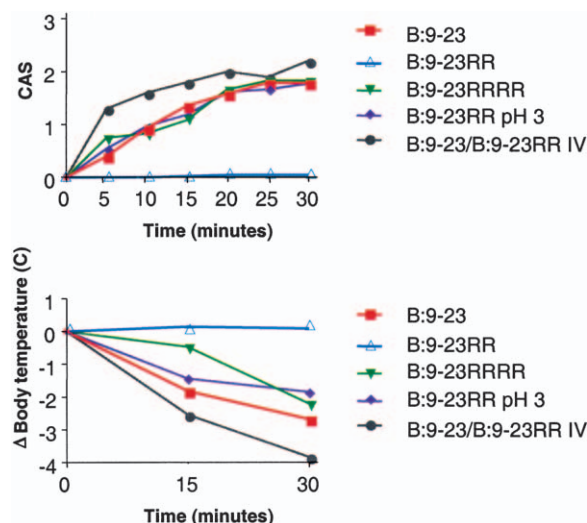


FIG 5. **A**, Mean clinical anaphylaxis scores (CAS) of sensitized mice challenged by means of subcutaneous injection of B:9-23, B:9-23RR, and B:9-23RR at a pH of less than 5 or B:9-23RRRR or intravenous injection of either B:9-23 or B:9-23RR. **B**, Mean core body temperatures of sensitized mice in the same experiments shown in Fig 4, **A**.

TABLE II. Summary of anaphylaxis induction in sensitized NOD mice by using B:9-23 compared with B:9-23RR, B:9-23RR given at pH 3, and B:9-23RRRR

	B:9-23, 100 μ g/dose, pH 7	B:9-23RR, 100 μ g/dose, pH 7	B:9-23RR, 100 μ g/dose, pH 3	B:9-23RRRR, 100 μ g/ dose, pH 7
Number anaphylaxis	67/69	2/45	17/22	11/12
Percent anaphylaxis	97%	4%	77%	92%

Only B:9-23RR given at a neutral pH (equal to pl) is able to prevent anaphylaxis.

23 (pl 7) in neutral solution (pH 7) into physiologic tissue (pH 7.4) will enhance peptide aggregation while preserving immunogenicity. Similarly, increasing the pI of B:9-23 to become basic with the addition of 4 arginine residues (B:9-23RRRR [pI 10.6]) also increases solubility and induced anaphylaxis in 11 of 12 sensitized mice when injected at neutral pH. A summary of the results of the anaphylaxis experiments with modified peptides is shown in Table II. Mean anaphylaxis scores and core body temperatures are shown for all groups of immunized mice after challenge in Fig 5.

To determine whether vaccination with the modified B:9-23RR peptide was able to protect against diabetes, we evaluated the effects of immunization with B:9-23RR in NOD mice (Fig 6, **A**). Mice injected subcutaneously with 10 μ g/wk B:9-23, B:9-23RR, or a control peptide, TT:830-843, were evaluated for 52 weeks. By means of survival analysis, the antidiabetic effect of B:9-23RR was similar to that of B:9-23 (not significantly different,

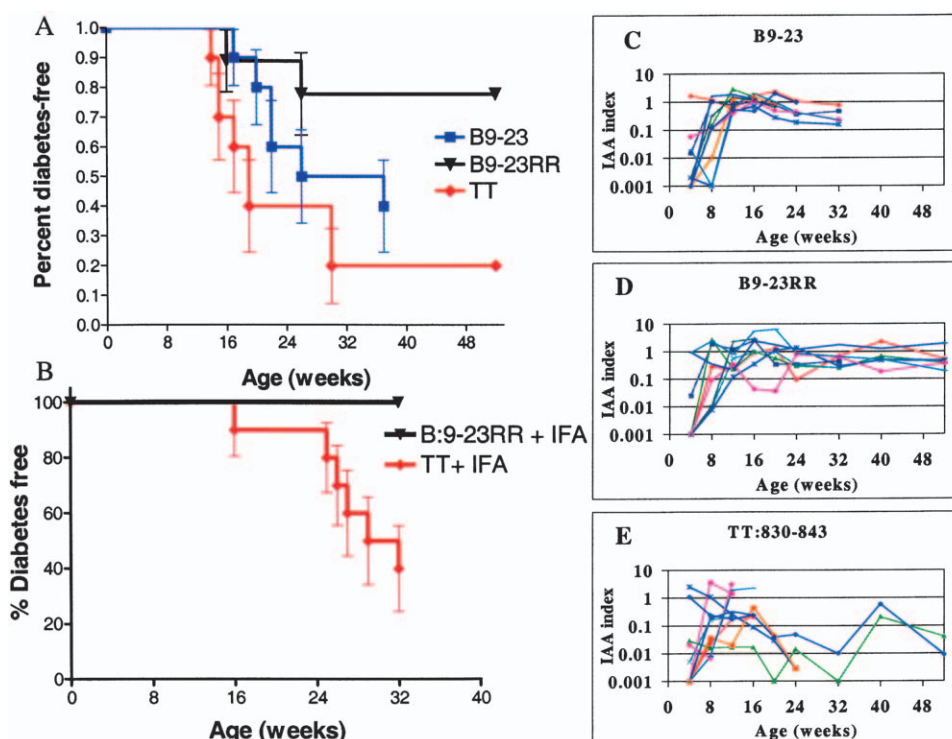


FIG 6. A, Survival analysis for NOD female mice receiving continuous weekly immunizations of B:9-23, B:9-23RR, or TT:830-843 in saline. B:9-23RR (10 µg per dose) protects NOD female mice against diabetes. It is superior to TT:830-843 (control) peptide but not significantly different from B:9-23 at the same dose. B, Survival analysis for NOD female mice receiving B:9-23RR and TT:830-843 (100 µg per dose) in incomplete Freund adjuvant starting at 15 weeks of age. Even in prediabetic mice, the effect of B:9-23RR was sufficiently robust to halt the development of diabetes. C-E, Induction of anti-insulin autoantibodies (IAAs) after peptide immunization. IAA induction is similar for mice receiving B:9-23 or B:9-23RR but higher than for mice receiving TT:830-843 peptide. Each data set represents a single mouse followed over time.

$P = .13$) and superior to that of TT:830-843 ($P < .02$). An additional protection study comparing a single dose of B:9-23RR or TT:830-843 in incomplete Freund adjuvant given subcutaneously at 15 weeks of age also confirms significant protection ($P < .01$) against diabetes, even in prediabetic mice (Fig 6, B). Furthermore, both B:9-23 and B:9-23RR delivered in saline induce high levels of insulin autoantibodies in NOD mice compared with the spontaneous insulin autoantibodies seen in TT:830-843-immunized mice (Fig 6, C-E). Finally, in a separate experiment testing the efficacy of repeated high-dose (100 µg/dose) peptide in saline to continue to prevent anaphylaxis beyond the usual time to develop anaphylaxis, 10 female NOD mice each received either B:9-23RR, TT:830-843, or saline starting at 4 weeks of age until 16 weeks of age. Although none of the mice had anaphylaxis in this study, there was also no significant protection from diabetes between B:9-23RR and TT:830-843 or saline. Thus lower doses of peptide in saline or the use of an adjuvant might be necessary to induce diabetes protection. Differential responses to higher or lower doses of peptide have been observed by us and described by others during peptide immunization strategies to develop tolerogenic T cells.^{13,14}

DISCUSSION

Anaphylaxis has been reported with several types of peptide immunization.³⁻⁸ It is unclear as to how a peptide only 15 amino acids long could cause cross-linking and activation of mast cells and basophils. In fact, current evidence suggests that peptide therapy might be safer than whole protein for immunotherapy.¹⁵ One possibility is that disulfide bonds occur between cysteine residues within our peptide, causing peptide dimers. However, Pedotti et al⁵ observed similar anaphylactic responses in experimental autoimmune encephalitis to a PLP peptide, which is only 13 amino acids long and does not contain cysteine residues.

There is currently no standard clinical test to determine who will become sensitized to a peptide and when anaphylaxis might occur. Thus an immunization strategy that would minimize the chance of developing anaphylaxis in response to peptide injection would be valuable. On the basis of our preliminary observations that rapid systemic delivery of peptide potentially induces anaphylaxis in sensitized mice, we hypothesized that slowing the rate of absorption of a peptide would prevent anaphylaxis. For example, in sensitized mice, even 1 µg of B:9-23 injected

intravenously can induce anaphylaxis, whereas 100 μ g is generally required when injected subcutaneously. We hypothesized that altering the pI of the peptide to neutral could prevent anaphylaxis by decreasing solubility at neutral pH, without necessarily affecting immunogenicity. The native peptide B:9-23 (pI 5.4) induces anaphylaxis, whereas the C-terminally modified peptide B:9-23RR (pI 7.0) avoids anaphylaxis, even though both peptides can induce anaphylaxis when injected intravenously. This protection with subcutaneous injection is dependent on the balance between peptide pI and solution pH. A peptide in a pH identical to its pI has the least net charge (equal positive and negative charges) and thus tends to aggregate in solution. As a peptide is brought to a pH further from the pI, a net charge develops that increases the peptide's solubility. Thus the change from B:9-23 (pI 5.4) to B:9-23RR (pI 7.0) causes the modified peptide to aggregate when delivered to subcutaneous tissue. Supporting this, B:9-23RR given at a pH of less than 5 will induce anaphylaxis, as does B:9-23RRRR (pI 10.6) given in a neutral solution. The fact that B:9-23RRRR does not also protect from anaphylaxis suggests that simply modifying the C-terminus with the addition of 2 arginine molecules is not responsible for anaphylaxis protection. In addition, modification of the C-terminus in this case does not alter immunologic properties of the peptide. B:9-23RR retains the ability to prevent diabetes in female NOD mice on repeated subcutaneous injection and induces insulin autoantibodies comparable with B:9-23.

Slowing the rate of absorption of a drug to alter pharmacokinetics has precedents. One example of a drug that has a modified pI to delay release is insulin Glargine (Lantus, Aventis Pharmaceuticals, Bridgewater, NJ), in which 2 arginine amino acids are added to the C-terminus of the B chain of insulin, increasing the pI to neutral.¹⁶ Glargine is delivered in an acidic solution (pH 4) in which it is completely soluble. On injection into subcutaneous tissue, the solution is buffered, and the drug microprecipitates under the skin, leading to crystallized drug that is slowly released. Decreasing the intravenous infusion rate of protein drugs, such as infliximab, is occasionally used to avoid infusion reactions. Additional ways to delay the release profile of a drug include the use of ion pair formation,^{16,17} polymeric microspheres in depot injections,¹⁸ or reverse thermosponsive gel systems.¹⁹ However, these drug delivery vehicles affect immunogenicity in complex ways. The ability to deliver peptide vaccines in saline alone might be advantageous.

In our model of self-peptide anaphylaxis, we have previously demonstrated that both histamine and PAF are involved and mediated through IgE and IgG. Although these mice have high titers (detectable at dilutions of 1 in a million) of anti-peptide IgG1 antibodies, we have been unable to detect peptide-specific IgE antibodies. This might be due to the lack of a sensitive assay or interference by high levels of circulating peptide-specific IgG1 antibodies. The involvement of IgE in this system is of relevance because anaphylaxis in human subjects involves

primarily IgE and histamine, with no evidence to date to support the role of IgG or PAF.²⁰

The mechanism underlying protection from anaphylaxis by slowing the absorption of peptide is not known. After antigen exposure, the release of preformed histamine from granules is generally complete within 15 to 30 minutes. It is possible that limiting the amount of peptide available per unit of time results in fewer mast cells and basophils becoming cross-linked and subsequently activated. However, it is more likely that all cells participate, but suboptimal peptide availability leads to less degranulation and release of preformed mediators.²¹ The concept of desensitization should also be considered after B:9-23RR injection. Downregulatory events might participate in limiting the amount of histamine release in response to antigen. In single-cell experiments desensitization occurs within 15 minutes of antigen exposure that is dependent on the absence of intracellular calcium. The possibility of differential T-cell reactivity to B:9-23 versus B:9-23RR (eg, by anergy or deletion) is not likely because both peptides can sensitize, and both peptides given intravenously can induce anaphylaxis. Thus the mechanism of B:9-23RR injection leading to protection from anaphylaxis is likely to be very complex.

In summary, we have developed a method to alter the pI of a peptide to prevent anaphylaxis during immunotherapy, while retaining desired immunologic properties. This might have general applicability as a technique to decrease risk of anaphylaxis without decreasing immunogenicity or therapeutic effect.

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