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Immunology

Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9–23)

DYLAN DANIEL*† AND DALE R. WEGMANN*‡§¶

*Barbara Davis Center for Childhood Diabetes, †Interdepartmental Program of Immunology, and Departments of §Immunology and ‡Pediatrics, University of Colorado Health Sciences Center, Denver, CO 80262

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ABSTRACT **The observation that overt type I diabetes is often preceded by the appearance of insulin autoantibodies and the reports that prophylactic administration of insulin to biobreeding diabetes-prone (BB-DP) rats, nonobese diabetic (NOD) mice, and human subjects results in protection from diabetes suggest that an immune response to insulin is involved in the process of beta cell destruction. We have recently reported that islet-infiltrating cells isolated from NOD mice are enriched for insulin-specific T cells, that insulin-specific T cell clones are capable of adoptive transfer of diabetes, and that epitopes present on residues 9–23 of the B chain appear to be dominant in this spontaneous response. In the experiments described in this report, the epitope specificity of 312 independently isolated insulin-specific T cell clones was determined and B-(9–23) was found to be dominant, with 93% of the clones exhibiting specificity toward this peptide and the remainder to an epitope on residues 7–21 of the A chain. On the basis of these observations, the effect of either subcutaneous or intranasal administration of B-(9–23) on the incidence of diabetes in NOD mice was determined. The results presented here indicate that both subcutaneous and intranasal administration of B-(9–23) resulted in a marked delay in the onset and a decrease in the incidence of diabetes relative to mice given the control peptide, tetanus toxin-(830–843). This protective effect is associated with reduced T-cell proliferative response to B-(9–23) in B-(9–23)-treated mice.**

It is now well established that insulin-dependent diabetes mellitus (IDDM) is an autoimmune disorder in which the insulin-producing beta cells are specifically destroyed. Although the available data indicate that T cells are the predominant mediators of beta cell destruction (1–4), overt diabetes is often preceded by the appearance of circulating antibodies specific to a number of beta cell products, among which is insulin (5). Insulin autoantibodies (IAAs) have been found in many new onset diabetic subjects prior to the administration of exogenous insulin (5) and have been found to precede the onset of diabetes by many years in certain individuals. The nonobese diabetic (NOD) mouse, which develops IDDM with many similarities to the human disease, is considered to be a good model of type I diabetes (6, 7), and IAAs have also been found to be present in NOD mice prior to the onset of diabetes (8–10). The presence of IAAs implies that there is also a T-cell response to this antigen, and although NOD mice can respond to insulin when immunized with insulin (11), attempts to demonstrate spontaneous T-cell responses to insulin in NOD mice have failed (12). In contrast, results from our laboratory indicate that T-cell responses to insulin arise spontaneously in NOD mice but that insulin-specific T cells are sequestered within the islet infiltrates characteristic of the disease process in these mice (13). More detailed investigations have revealed that insulin-specific T cells are present in islet infiltrates from the early stages of infiltration until development of diabetes, that helper T1 (T_H1)-like cells dominate in this response (14), and that insulin-specific T cell clones can mediate the adoptive transfer of diabetes (15). One striking observation concerning the spontaneous T-cell response to insulin in NOD mice was the apparent antigenic dominance of residues 9–23 of the B chain [B-(9–23)] despite the fact that there was considerable T-cell receptor diversity among B-(9–23)-specific clones (15).

Other investigators have reported that insulin, when given to NOD mice either orally or subcutaneously, leads to a decrease in the incidence of diabetes (16, 17). More recently, it has been demonstrated that the isolated insulin B chain, when given subcutaneously in incomplete Freund's adjuvant (IFA), confers protection essentially equivalent to that observed with intact insulin (18, 19). The activity of the B chain in protecting NOD mice from diabetes and the findings that the spontaneous T-cell response to insulin is predominantly directed toward B-(9–23) suggest that the protective effect of insulin or B chain is mediated by modulation of insulin B-(9–23)-specific T cells. In this report, the antigenic dominance of B-(9–23) in the spontaneous T-cell response to insulin was examined by determining the epitope specificity of 312 insulin-specific T-cell clones that were independently derived from islet infiltrates of 21 NOD mice of various ages. In addition, the effect of administration of B-(9–23) by either subcutaneous or intranasal routes on the incidence of diabetes was examined. The results presented here indicate that B-(9–23) is the dominant epitope, with 93% of all insulin-specific clones tested responding to B-(9–23). However, a second epitope located on residues 7–21 of the A chain [A-(7–21)] has been identified, and all of the clones that were not B-(9–23) specific were found to be A-(7–21) specific. The antigenic dominance of B-(9–23) led to attempts to prevent development of diabetes by administration of B-(9–23). The results of these experiments indicated that potent protection from diabetes is conferred by subcutaneous administration of B-(9–23) in IFA and that intranasal exposure of NOD mice to B-(9–23) also resulted in marked protection from diabetes.

MATERIALS AND METHODS

Mice. NOD/bdc mice were obtained from the colony at the Barbara Davis Center and housed under specific pathogen-free conditions.

Antigens. Reverse-phase HPLC-purified insulin peptides A-(7–21) and B-(9–23) (SHLVEALYLVCGERG) and tetanus toxin (TT) peptide [residues 830–843; TT-(830–843), (QYIKANSKFIGITE)] (20, 21) were obtained for immunization and intranasal administration to prediabetic NOD mice (Molecular Resources Center, National Jewish Hospital, Denver).

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Abbreviations: NOD, nonobese diabetic; IDDM, insulin-dependent diabetes mellitus; IAAs, insulin autoantibodies; TT, tetanus toxin; IFA, incomplete Freund's adjuvant; CFA, complete Freund's adjuvant; T_H cell, helper T cell; ANOVA, analysis of variance.

¶To whom reprint requests should be addressed.

Establishment of Insulin-Specific T-Cell Clones. All of the insulin-specific T-cell clones described in Table 1 were obtained by limiting-dilution cloning of T-cell lines established from islet-infiltrating populations by *in vitro* stimulation with either porcine insulin (25 μg/ml) or irradiated NOD islet cells (2.0×10^4 cells per culture) in the presence of irradiated NOD spleen cells and lymphokine, as described (13). Islets were obtained from female NOD mice by collagenase digestion of the pancreas followed by manual isolation of individual islets. Infiltrating cells were released from the islets by mild trypsin digestion. The insulin-specific T-cell lines and clones were propagated by serial stimulation in the presence of NOD spleen cells, lymphokine, and porcine insulin (25 μg/ml) as described (13). All of the clones listed in Table 1 were tested at least twice in proliferation assays to confirm antigen specificity and have been cryopreserved for future use.

Immunization of NOD Mice with B-(9–23) or TT-(830–843). Mice were immunized with 50 μg of either B-(9–23) or TT-(830–843) emulsified in complete Freund's adjuvant (CFA) (50-μl total volume) at the base of the tail (22). After at least 7 days postimmunization, the draining lymph nodes (inguinal and periaortic) were removed, and cell suspensions were prepared. These cell suspensions were washed and cultured in triplicate or quadruplicate at 1.0×10^6 cells per well in 96-well microculture plates in Click's medium supplemented to either 0.5% with normal mouse serum or 1% with Nutridoma SP (Boehringer Mannheim) and the indicated peptide as antigen. These cultures were incubated at 37°C for 5 days with a pulse of 0.5 μCi of [^{3}H]dTTP (1 Ci = 37 GBq) 18 h prior to harvest.

Subcutaneous Administration of B-(9–23) or TT-(830–843) to Prediabetic NOD Mice. Four-week-old female NOD/bdc mice were divided into experimental and control groups. The mice were injected once subcutaneously with 100 μg of either B-(9–23) or TT-(830–843) in IFA. The blood glucose levels of the mice were monitored weekly by using an ExacTech Blood Glucose Sensor (MediSense, Waltham, MA), and mice were considered to be diabetic after three consecutive blood glucose values above 10 mM.

Intranasal Administration of B-(9–23) or TT-(830–843) to Prediabetic NOD Mice. Four-week-old female NOD/bdc littermates were divided into experimental and control groups. As described previously (23), the mice were lightly anesthetized and administered 40 μg of peptide in 20 μl of phosphate-buffered saline (PBS; 10 mM sodium phosphate, pH 7.4/0.14 M sodium chloride) (20 μg in 10 μl per nostril) intranasally on three consecutive days. The experimental group was given insulin peptide B-(9–23) and the controls were given TT-(830–843). The mice were monitored for diabetes as described above.

RESULTS

Antigenic Dominance of B-(9–23) in the Spontaneous T-Cell Response to Insulin and Identification of A-(7–21) as a Subdominant Epitope. In a previous report, it was observed that six of six insulin-specific T-cell clones tested responded to peptide B-(9–23) and that a number of uncloned, insulin-specific T-cell lines responded only to B-(9–23) suggesting that B-(9–23) contained the dominant epitopes for this response (15). We have now analyzed the epitope specificity of 312 insulin-specific T-cell clones established from lines initiated from the islet infiltrates of 21 female NOD mice. The results of this analysis (Table 1) reveal a number of characteristics of the spontaneous T-cell response to insulin. First, insulin B-(9–23)-specific T cells are present in islet infiltrates from 4 weeks of age until 12 weeks of age. Second, B-(9–23) is the overwhelmingly dominant epitope in this response, with all but 22 of the 312 (93%) clones tested being B-(9–23) specific. Third, a second epitope in this response has been identified, and the 22 clones that were not B-(9–23) specific recognized epitopes present on a synthetic peptide comprising residues 7–21 of the A chain [A-(7–21)]. It is of interest that B-(9–23) is dominant in this response, not only with respect to the percentage of clones that are B-(9–23) specific but also with regard to the proportion of mice that exhibit a detectable response to B-(9–23). Thus, all of the A-(7–21)-specific clones originated from 3 of the 21 mice examined, whereas all 21 mice yielded B-(9–23)-specific clones. Despite the overall dominance of B-(9–23), the results suggest that A-(7–21)-specific T cells can dominate the response in individual mice, such as in line PD7-7, where 16 of 21 clones tested responded to A-(7–21).

Table 1. Epitope specificity of insulin-specific T-cell clones

T-cell line	Age of donor, weeks	No. of clones		
		Tested	B-(9–23) specific	A-(7–21) specific
PD4-1	4	22	22	0
PD4-7	4	24	24	0
PD4-9	4	24	24	0
PD5-3	5	6	6	0
PD5-4	5	5	5	0
PD5-9	5	21	21	0
PD6-4	6	17	17	0
PD6-6	6	9	9	0
PD6-10	6	11	11	0
PD6-11	6	7	7	0
PD6-12	6	39	39	0
PD7-2	7	2	2	0
PD7-7	7	21	6	15
PD9-7	9	9	6	3
PD9-8	9	24	24	0
PD9-9	9	24	20	4
PD11-4	11	24	24	0
PD12-1	12	2	2	0
PD12-2	12	7	7	0
PD12-3	12	1	1	0
PD12-4	12	13	13	0

Insulin-specific T-cell clones were established from the islet-infiltrating cells of 21 individual NOD mice by limiting dilution cloning of insulin-specific lines. The epitope specificity of the clones was determined by screening a panel of five overlapping peptides based on the primary structure of murine insulin II. The majority of clones were specific for the B chain peptide B-(9–23), while a minority were specific for an A chain peptide A-(7–21).

The potential dominance of A-(7–21) in the insulin response of certain mice made it of interest to determine if A-(7–21)-specific clones had *in vivo* and *in vitro* properties similar to those of B-(9–23)-specific clones. The proliferative responses of several of the A-(7–21)-specific T-cell clones (not shown) indicate that all clones responded both to insulin and to A-(7–21) with dose–response curves similar to those of B-(9–23)-specific clones. The B-(9–23)-specific clones were found to be capable of adoptive transfer of diabetes to either young NOD mice or NOD *scid* mice (15). To assess the diabetogenicity of A-(7–21)-specific T cells, two independently derived A-(7–21)-specific clones, one from each of the two 9-week-old mice from which these clones were isolated, were injected into 12-day-old NOD mice, and the blood glucose levels of the mice were monitored. The results of this experiment (not shown) indicate that both of these clones were able to accelerate diabetes in young NOD mice. These cells, therefore, have the potential to be pathogenic and may participate in beta cell destruction in those mice which mount a response to this epitope.

Primed Lymph Node Proliferative Response of NOD Mice to B-(9–23) and TT-(830–843). Identification of B-(9–23) as the dominant epitope in the spontaneous T-cell response to insulin provides a defined reagent for use in attempts at antigen-specific therapy. The peptide TT-(830–843) was selected as a control antigen for these investigations. The T-cell responsiveness of NOD mice to B-(9–23) and TT-(830–843)

(sequences shown in *Materials and Methods*) was determined by primed lymph node proliferation assays. The results of a typical experiment are shown in Fig. 1, where it can be seen that NOD mice mount vigorous T-cell responses to both of these peptides. These results confirm that TT-(830–843) is an appropriate control antigen for these investigations.

Protection of NOD Mice from Diabetes by a Single Subcutaneous Injection of B-(9–23) in IFA. It has been reported that subcutaneous administration of either intact insulin or the isolated B chain of insulin emulsified in IFA reduces the incidence of diabetes in NOD mice (18, 19). To address the question as to whether B-(9–23) has an effect similar to that observed with the B chain, 4-week-old, female, littermate NOD mice were given a single subcutaneous injection of 100 μg of either B-(9–23) or TT-(830–843) emulsified in IFA. These mice were then monitored for development of diabetes by measurement of blood glucose levels. The results of this experiment (Fig. 2) indicate that mice which received B-(9–23) had a greatly reduced incidence of diabetes relative to TT-(830–843)-treated mice ($P = 0.0055$ by Fischer's exact test) and to the incidence in the colony (not shown) ($P < 0.00001$ by Fischer's exact test).

Protection of NOD Mice from Diabetes by Multiple Intranasal Treatments with B-(9–23). The powerful protective effect obtained by administration of B-(9–23) in IFA led to investigation of a less invasive route of administration, intranasal instillation (23). The experiments described here involved dividing female mice within litters into experimental and control groups which were given either B-(9–23) or TT-(830–843) at 4 weeks of age. The mice were treated again at 9 weeks of age and at 4-week intervals thereafter and were monitored for diabetes. The results of this experiment (Fig. 3) indicate that mice treated with B-(9–23) were protected from diabetes, while recipients of TT-(830–843) were not. Although one of the B-(9–23) recipients developed diabetes, it is clear that there is a dramatic delay in the onset of diabetes in B-(9–23)-treated mice relative to that observed in mice that received TT-(830–843) ($P = 0.0236$ by log-rank test).

Reduction in the Primed Lymph Node Response to B-(9–23) in Mice Given B-(9–23) via the Intranasal Route. Other investigators have observed that intranasal administration of peptide antigens results in an antigen-specific decrease in the proliferative response of primed lymph node cells (23). To begin to define the mechanism by which intranasally administered B-(9–23) protects NOD mice from IDDM, the T-cell proliferative responses of control mice or mice treated with either B-(9–23) or TT-(830–843) were determined. In these experiments, mice were treated for 5 consecutive days (200 μg of antigen total) rather than the 3-day regime used in the protection experiments because the protection experiments involve multiple periodic treatments and the proliferative responses were assessed after a single 5-day treatment. The results of a typical experiment are shown in Fig. 4, where it can be seen that proliferative responses to B-(9–23) are markedly lower ($P < 0.0001$ by ANOVA test) in mice that received intranasal B-(9–23) relative to those of untreated control mice or mice given TT-(830–843). These results indicate that intranasal administration of B-(9–23) results in a reduction in T-cell responsiveness to B-(9–23).

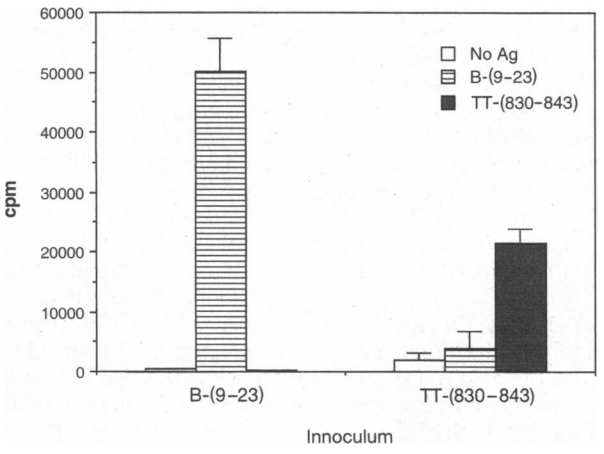

FIG. 1. Lymph node proliferation assay of NOD mice immunized with B-(9–23) or TT-(830–843) emulsified in CFA. Responses of inguinal and periaortic lymph node cells (1.0×10^6) isolated from mice immunized with 50 μg of either B-(9–23) or TT-(830–843) and stimulated *in vitro* with 33 μg of either B-(9–23) (17.4 μM) or TT-(830–843) (17.9 μM) per ml were determined as described in *Materials and Methods*. The cultures were incubated for 5 days with a pulse of 0.5 μCi of [^{3}H]dTTP added to each well 18 h prior to harvest. The results of a representative experiment are expressed as mean cpm for triplicate or quadruplicate wells.

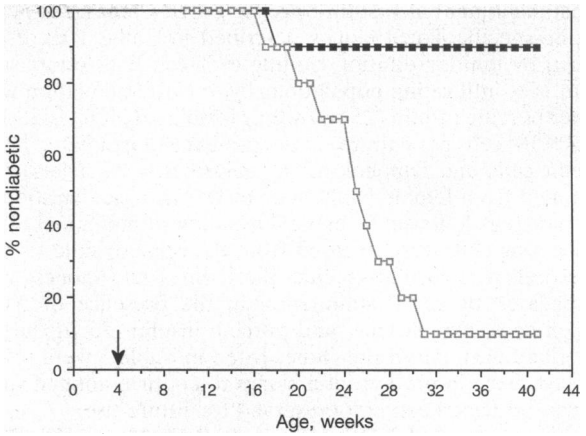

FIG. 2. Effect of subcutaneous injection of insulin peptide B-(9–23) in IFA on diabetes incidence in NOD mice. Four-week-old female NOD mice that received subcutaneous B-(9–23) + IFA ($n = 10$) (■) show delayed onset ($P = 0.0064$ by Mann–Whitney test) and reduced frequency ($P = 0.0055$ by Fisher's exact test) of diabetes when compared with mice that received subcutaneous TT-(830–843) + IFA ($n = 10$) (□). Arrow indicates time of subcutaneous injection.

DISCUSSION

Type I diabetes in NOD mice and in human subjects is the clinically apparent endpoint of a chronic autoimmune re-

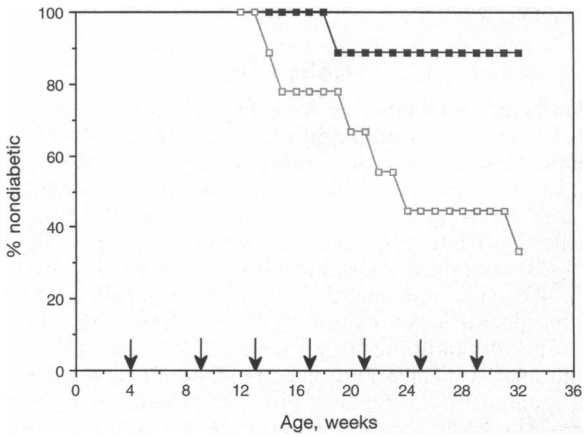

FIG. 3. Effect of multiple intranasal doses of insulin peptide B-(9–23) on diabetes incidence in NOD mice. Four-week-old female NOD mice that received intranasal B-(9–23) ($n = 9$) (■) every 4 or 5 weeks show delayed onset of diabetes ($P = 0.0236$ by log-rank test when compared with littermates that received intranasal TT-(830–843) ($n = 9$) (□). Arrows indicate time of intranasal treatments.

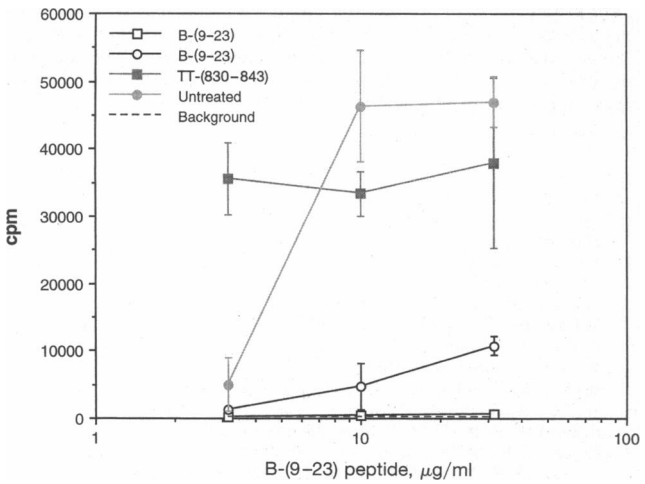

FIG. 4. Effect of intranasal administration of insulin peptide B-(9–23) in CFA primed proliferation responses to B-(9–23). Six-week-old female NOD/bdc mice were intranasally administered 40 μg of either B-(9–23) or TT-(830–843) or left unmanipulated as control mice on 5 consecutive days (200 μg total). One week after the last intranasal treatment, the mice were immunized with 50 μg of B-(9–23) in CFA at the base of the tail. One week after immunization, the lymph node cells were tested for proliferation in response to B-(9–23) peptide, as described in *Materials and Methods*. The results of a representative experiment are expressed as mean cpm for triplicate cultures. Two mice treated intranasally with B-(9–23) (open symbols) showed reduced proliferative responses to 33 μg of B-(9–23) per ml (17.4 μM) [$p < 0.0001$ by analysis of variance (ANOVA) test] and 10 μg of B-(9–23) per ml (5.3 μM) ($P < 0.0001$ by ANOVA test) when compared with littermates treated with TT-(830–843) or untreated mice (closed symbols). Untreated and TT-(830–843)-treated mice showed similar proliferative responses at 33 μg and 10 μg of B-(9–23) per ml (no statistical difference by ANOVA test). All groups showed comparable responses to 1 μg of concanavalin A per ml (not shown).

sponse in which the islet beta cells are specifically destroyed (7, 24, 25). The chronic nature of this process is illustrated by the observations that both NOD mice and human subjects exhibit autoantibody responses to a number of beta cell antigens a considerable length of time prior to the onset of symptoms (26–29). The presence of these autoantibodies, especially the appearance of antibodies specific to multiple defined beta cell products, provides a reliable means of identification of human subjects likely to develop IDDM (30, 31). In addition, the lag between the initial appearance of circulating autoantibodies and destruction of sufficient beta cell mass to result in insulin dependence provides a window of time in which to intervene in the disease process. T cells are the predominant mediators of beta cell destruction, and there are numerous reports in which it has been demonstrated that experimental manipulations and agents that modulate T-cell function protect NOD mice from diabetes (7, 32). Although general immunosuppressive agents have been demonstrated to provide some protection from diabetes, it is clear that targeting only those cells that are islet cell specific is the approach of choice. With regard to this, it has been observed that administration of glutamic acid decarboxylase 65 (33, 34), heat shock protein 65 (35), porcine insulin (16, 17), or the isolated B chain of porcine insulin (19) to young NOD mice can confer protection from IDDM.

The results of investigations of the spontaneous T-cell response to insulin in NOD mice provided evidence for participation of insulin-specific T cells in beta cell damage (14, 15). Two features of this response are of note with regard to utilization of insulin-specific T cells as targets for antigen-specific intervention. First, insulin-specific T cells have been found to be present in islet infiltrates at relatively high frequencies essentially throughout the course of progression of beta cell damage (14). Second, the results presented here, as well as in previous reports, indicate that this response is directed almost entirely toward the 15-residue peptide B-(9–23) (14, 15). In the present analysis, 290 of 312 insulin-specific clones isolated from 21 individual NOD mice of various ages were found to be B-(9–23) specific. These results confirm the results of previous analyses and also indicate that the antigenic dominance of B-(9–23) persists from early in the disease process until the time of onset of IDDM. These characteristics provide a population of T cells specific to a defined peptide antigen that are available for targeting over a broad window of time during disease progression, and the reports in which administration of insulin was shown to provide protection from diabetes (16, 17, 19) suggest that this is a promising approach.

These results demonstrate that subcutaneous injection of B-(9–23) in IFA provides the most potent protection from diabetes, with 90% of B-(9–23) recipients remaining diabetes free out to 1 year of age. In contrast, the TT-(830–843) recipients developed diabetes at a rate that paralleled the incidence in the NOD/bdc colony, with 90% being diabetic by 31 weeks of age. The present results concerning the effect of subcutaneous administration of B-(9–23) suggest that B-(9–23) is more effective than either intact insulin or the isolated B chain, in that multiple periodic injections were required in previous studies (16, 19) whereas only a single injection was given in these investigations. Additionally, the fact that B-(9–23) provides similar if not greater protection than intact insulin or the B chain suggests that these latter two agents also exert their effects via B-(9–23)-specific T cells.

A less invasive route of exposure of experimental animals and man to antigens that has been demonstrated to lead to modulations in T-cell responses (23) and to amelioration of induced autoimmune disorders (36–38) is via inhalation or exposure to the oral or nasal mucosal surfaces. It has been speculated that this response evolved as a means of preventing undesired hypersensitivities to environmental agents (39). The effect of intranasal instillation of B-(9–23) on the development of diabetes in NOD mice has been determined, and the results of a number of experiments were consistent, with protection being observed in each case. The experiments described here involved treatment of female NOD mice at 4 weeks of age with subsequent treatments at 4-week intervals, but protection from IDDM has also been observed after a single treatment given at 12 weeks of age and after treatment of mice starting at 8 weeks of age (results not shown). It thus appears that B-(9–23), when given by intranasal instillation, can protect NOD mice from IDDM when treatment is started at early, intermediate, or late stages of beta cell destruction.

At this time, there is little information on the mechanism by which administration of B-(9–23) via the subcutaneous or intranasal routes confers protection from diabetes, nor is it clear that these two routes of administration have a common mechanism. The role of cytokines in autoimmune disorders, specifically the cytokines produced by T_H1 versus T_H2 T cells, has been the subject of recent interest, and it has been suggested that T_H1-like cells are responsible for destruction, whereas T_H2-like cells may provide protection (40). Supporting this type of mechanism for beta cell destruction in NOD mice are the observations that NOD mice treated subcutaneously with either intact insulin or the B chain in IFA, a protocol similar to that used here, have a dramatically reduced incidence of IDDM that is associated with decreased production of interferon γ (IFN-γ) and increased production of interleukin 4 (IL-4) within the islet infiltrates (19). Additional evidence for involvement of cytokines comes from the observation that oral administration of antigens, which leads to protection in induced models of autoimmunity, can result in a substantial change in the ratio of T_H1-associated cytokines to T_H2-associated cytokines (41). Although we have not quantified cytokine production in the islet infiltrates of mice treated with

B-(9–23) via the subcutaneous route, it seems likely that this involves the same mechanism as observed with insulin or B chain.

With regard to the mechanism of protection of intranasal instillation, it has been suggested that modulation of antigen-presenting function by alveolar macrophages results in the induction of regulatory T cells (39), possibly of the γ/δ phenotype (42). It should be noted that the amount of B-(9–23) required for intranasal protection as described here was far less than was required for oral-tolerance-mediated protection of NOD mice from IDDM (17), making it unlikely that this effect is due to ingestion of B-(9–23) and induction of oral tolerance. The antigenic dominance of B-(9–23) for insulin-specific T cells within islet-infiltrating populations implicates B-(9–23)-specific T cells as the target population for this effect. The results of the experiments in which mice were treated with B-(9–23) by intranasal instillation and subsequently challenged by immunization with B-(9–23) in CFA at the base of the tail indicate that B-(9–23)-treated mice have a dramatically reduced proliferative response to B-(9–23). It is likely that regulatory cells are induced in the draining lymph nodes and migrate to the islets where they exert the observed protective effect. In support of this concept, experiments in which cells from the cervical lymph nodes of mice given intranasal B-(9–23) were cultured in the presence of B-(9–23) indicate that there is a pronounced T_H2-like cytokine production by these populations upon stimulation with B-(9–23). This T_H2-like cytokine production profile is also reflected in analyses performed on B-(9–23)-specific T-cell clones obtained from these populations (unpublished observations). In contrast, numerous attempts to isolate T_H2-like, B-(9–23)-specific T cells from untreated mice by using conditions that should favor the outgrowth of these cells (anti-IFNγ and recombinant IL-4) have yielded negative results (unpublished observations). The *in vivo* activities of the T_H2-like cells derived from the draining lymph nodes are not known at this time.

One issue of obvious interest is the applicability of these observations to human subjects. Although IAAs were first observed in newly diabetic human subjects (5), have been demonstrated to precede overt diabetes by many years, and are considered to be predictive of a "prediabetic" state (27), the T-cell responsiveness to insulin of IAA-positive subjects has not been measured in a systematic fashion. It has been reported that newly diabetic subjects exhibited T-cell responses to insulin before treatment with insulin was initiated, but these results have not been confirmed (43). The results of a pilot trial suggest that administration of insulin to subjects considered to be at risk for IDDM resulted in significant protection from development of diabetes (44), but this involved relatively small group sizes and awaits confirmation. Results from NOD mice indicate that insulin-specific T cells are preferentially sequestered within the islet infiltrates and that peripheral responses are not readily detectable. These observations render any results obtained by using human peripheral blood of questionable relevance to the disease process. However, results obtained from peripheral blood T cells may provide information on the most important issue, namely whether B-(9–23) is a T-cell epitope in DR3/4-positive individuals.

In summary, an extensive analysis of the spontaneous T-cell response to insulin in NOD mice indicates that B-(9–23) is the dominant epitope within islet-infiltrating cells and that treatment of NOD mice with B-(9–23) given either subcutaneously or via intranasal instillation confers potent protection from IDDM. This protective effect is associated with a general T-cell hyporesponsiveness to B-(9–23) in treated mice. Intranasal administration of insulin or insulin peptides may be of value for prevention of type I diabetes in human subjects.

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