

Aerosol Insulin Induces Regulatory CD8 $\gamma\delta$ T Cells That Prevent Murine Insulin-dependent Diabetes

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Summary

Cellular immune hyporesponsiveness can be induced by the presentation of soluble protein antigens to mucosal surfaces. Most studies of mucosa-mediated tolerance have used the oral route of antigen delivery and few have examined autoantigens in natural models of autoimmune disease. Insulin is an autoantigen in humans and nonobese diabetic (NOD) mice with insulin-dependent diabetes mellitus (IDDM). When we administered insulin aerosol to NOD mice after the onset of subclinical disease, pancreatic islet pathology and diabetes incidence were both significantly reduced. Insulin-treated mice had increased circulating antibodies to insulin, absent splenocyte proliferation to the major epitope, insulin B chain amino acids 9–23, which was associated with increased IL-4 and particularly IL-10 secretion, and reduced proliferation to glutamic acid decarboxylase, another islet autoantigen. The ability of splenocytes from insulin-treated mice to suppress the adoptive transfer of diabetes to nondiabetic mice by T cells of diabetic mice was shown to be caused by small numbers of CD8 $\gamma\delta$ T cells. These findings reveal a novel mechanism for suppressing cell-mediated autoimmune disease. Induction of regulatory CD8 $\gamma\delta$ T cells by aerosol insulin is a therapeutic strategy with implications for the prevention of human IDDM.

Insulin-dependent diabetes mellitus (IDDM)¹ results from the selective destruction of insulin-producing β cells in the islets of the pancreas, within an autoimmune inflammatory “insulinitis” lesion (1, 2). The primary role of autoreactive T cells in mediating β cell destruction has been shown directly in two spontaneous animal models of IDDM, the Bio-Breeding (3) rat and the nonobese diabetic (NOD) mouse (4). Target autoantigens that trigger or drive immune reactivity to β cells not only have diagnostic applications, but are potential agents for specific immunotherapy (5–8). Several potentially pathogenic islet/ β cell autoantigens have been identified by their reactivity with circulating antibodies or T cells in rodents and humans with subclinical or clinical IDDM, particularly insulin, glutamic acid decarboxylase (GAD), and a tyrosine phosphatase, IA-2 (9). Insulin and its precursor, proinsulin, however, are the only IDDM autoantigens that are β cell specific. Insulin autoantibodies are a risk marker for the development of clinical IDDM (10) and have been detected before autoantibodies to other islet antigens in the offspring of diabetic mothers (11). Increased proliferation of peripheral blood T cells to

human insulin can be demonstrated in up to half of subclinical and recently diagnosed IDDM subjects (12), but responses are relatively low. This is possibly because the dominant human T cell epitope is in proinsulin; a peptide that spans the natural cleavage site between the B chain of insulin and the connecting (C) peptide in proinsulin elicits T cell proliferation in a majority of subclinical subjects (13). In the NOD mouse, insulin autoantibodies are reported to be a risk marker for the development of diabetes (14) and the majority of T cell clones generated from the insulinitis lesion react to the insulin B chain, amino acids 9–23 (15).

An autoantigen can be assumed to be pathogenic if its administration modifies the natural history of autoimmune disease. Autoantigen-specific strategies of immune tolerance induction have been shown to favorably modify the natural history of experimental autoimmune disease in rodents (5–8, 16). The presentation of soluble protein antigen to mucosal surfaces, classically via the oral route, results in selective suppression of antigen-specific, T cell-mediated, delayed-type hypersensitivity (DTH) and IgE responses (16–18). “Oral tolerance” has been associated with the deviation of immunity away from T cell (Th1) to antibody (Th2) responses, with the induction of regulatory T cells and, at higher antigen doses, with both T cell anergy and T cell deletion (16, 19). Despite widespread interest in the possibility of preventing IDDM, relatively few studies have

¹Abbreviations used in this paper: DTH, delayed-type hypersensitivity; GAD, glutamic acid decarboxylase; IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic (mice).

evaluated mucosa-mediated tolerance in the natural NOD mouse model. Zhang et al. (20) found that oral porcine insulin (1 mg twice weekly) delayed the onset and reduced the incidence of diabetes, and was associated with splenic T cells that partially blocked the transfer of diabetes to young NOD mice by spleen cells from diabetic mice. Subsequently, Bergerot et al. (21) reported that the regulatory cells induced by oral insulin were CD4⁺ T cells. In studies of oral tolerance to guinea pig myelin basic protein in the Lewis rat model of experimental autoimmune encephalomyelitis (reviewed in reference 16), however, both CD4⁺ and CD8⁺ regulatory T cells that secrete IL-4, IL-10, TGF- β , or TGF- β , respectively, have been described. Daniel and Wegmann (15) recently reported that intranasal administration of insulin B chain amino acids 9–23 protected NOD mice from diabetes, but did not define a mechanism; Tian et al. (22) reported that a single intranasal dose of GAD T cell epitope peptides given to NOD mice before the onset of insulinitis induced the deviation of antiislet immunity towards Th2 responses and reduced diabetes incidence, in association with regulatory CD4, not CD8, T cells.

Holt and co-workers described the suppression of T cell and IgE responses to OVA after its inhalation in rats or mice, which was transferable by CD8⁺ T cells (18) and was subsequently found to be effected by small numbers of OVA-specific CD8 $\gamma\delta$ T cells (23). There is increasing evidence for the immunoregulatory role of $\gamma\delta$ T cells in general (24–29). We therefore decided to investigate the effect of aerosol insulin inhalation in the NOD mouse. This mode of delivery of autoantigen to the nasopharyngeal and upper bronchial mucosa could have advantages over oral insulin in humans. It is convenient and direct, might require a smaller dose, and be less likely to be associated with insulin degradation and variability of absorption. In particular, we wished to determine if aerosol insulin would be therapeutic if given after the onset of insulinitis and to define the nature of the regulatory cells that it might potentially induce.

Materials and Methods

Aerosol Treatment and Diabetes Assessment. Semisealed boxes of eight female NOD mice were each aerosolized by connection to a standard patient electric pump (Maymed Aerosol MKV; Anaesthetic Supplies, Sydney, Australia) and an Aeroflo nebulizer (Waite & Co., Sydney, Australia). Recombinant human insulin (Humulin R; Aza Research, West Ryde, Australia) or control OVA protein, at 4 mg/ml, was delivered over 10 min at an air flow rate of 6 liters/min in a rated droplet size of <5.8 μ m, to groups of 24–32 mice. All treatments were given between 0900 and 1100 h. Protocols and mouse care were approved and supervised by the institutional Animal Ethics Committee. Retroorbital venous blood was sampled at least every 28 d from 100 d of age, and mice were considered diabetic if their blood glucose, confirmed by a repeat test, was >11 mM. Glucose was measured with BM-Test Glycemic[®] strips and a Refolux[®] II meter (Boehringer Mannheim GmbH, Mannheim, Germany) on a drop of blood aspirated via a glass capillary tube from the retroorbital venous plexus of unanesthetized mice.

Histology. Mice were killed by CO₂ inhalation, and the pancreas and salivary glands were immediately removed into Bouin's fixative and embedded in paraffin. The insulinitis score, a measure of the severity of islet infiltration, was determined blindly by two independent investigators by grading and then averaging a minimum of 15 separate islets in serial 6- μ m pancreas sections stained with hematoxylin and eosin. The grading scale was as follows: 0, no infiltration, islet intact; 1, <10 periislet lymphoid cells, islet intact; 2, 10–20 peri- and intraislet lymphoid cells, islet intact; 3, >20 peri- and intraislet lymphoid cells, <50% of islet replaced or destroyed; 4, massive lymphoid infiltrate with >50% of islet replaced or destroyed. Infiltration of the salivary glands was graded by the number of lymphoid cells in clusters: 0, no cells; 1, <10 cells; 2, 10–50 cells; 3, >50 cells.

Immune Responses. Spleen cells from individual normoglycemic mice were treated with a red cell lysis buffer, resuspended, and incubated in quadruplicate at $2 \times 10^5/200 \mu$ l of serum-free HL-1 medium (Hycor, Portland, ME) containing 50 μ m 2-MER in round-bottom wells with the indicated concentrations of antigen. After 3 d at 37°C in 5% CO₂/air, 100- μ l aliquots from each replicate supernatant were collected and stored at -70°C for cytokine assays; the cells were then pulsed with [³H]thymidine, harvested 16 h later, and counted on a microscintillation counter (Topcount; Packard Instruments, Meriden, CT). Insulin was recombinant human (Humulin R; Eli Lilly), as used for aerosol treatments. Insulin B chain peptide corresponding to amino acids 9–23 of mouse insulin II (Peptide Express, Fort Collins, CO) was >90% pure, as determined by HPLC analysis. GAD65 was the recombinant human form expressed with a COOH-terminal hexahistidine in a baculovirus system and purified by Ni²⁺ chelation affinity chromatography. It was resolved as a single band in SDS-PAGE, and was endotoxin-free by the quantitative limulus lysate assay (BioWhittaker, Walkersville, MD).

IL-2, -4, -10, and IFN- γ were measured by ELISAs with mAb pairs (PharMingen, San Diego, CA); the lower limits of detection were 62, 16, 16, and 55 pg/ml, respectively. TGF- β 1 was measured with an ELISA kit (Promega, Madison, WI) with a lower limit of detection of 16 pg/ml.

To detect insulin antibodies, ¹²⁵I-labeled human insulin (~100,000 cpm; specific activity 120 μ Ci/ μ g) was incubated with or without excess unlabeled insulin (10 μ g/ml) in PBS containing a mixture of protease inhibitors and serial log dilutions of mouse serum for 5 d at 4°C. Complexes were then precipitated with rabbit anti-mouse globulin antiserum, washed, and counted in a gamma counter. Positive control sera (guinea pig antiporcine insulin serum, human IDDM sera) maximally precipitated 37–54% of the total radioactivity. Nonspecific binding, in the presence of excess unlabeled insulin, was \leq 3.3%.

Adoptive Transfer of Diabetes. Male NOD mice, 6–9 wk old (16 per group), were irradiated (800 rad) from a cobalt source, and 3–6 h later, received 2×10^7 pooled splenocytes from recently diabetic 14–19-wk-old female NOD mice, together with 2×10^7 splenocytes (or cells fractionated from this number) from either aerosol insulin- or OVA-treated mice, in 200 μ l via the tail vein. The onset of diabetes was then monitored by measuring blood glucose starting 2 wk after transfer.

Fractionation of Spleen Cell Populations. Spleen cells were treated with a red cell lysis buffer and resuspended in mouse tonic PBS. Total T cells were purified by non-adherence to nylon wool. CD4 and CD8 cells were positively selected/depleted magnetically with mAbs directly bound to MACS MicroBeads (Miltenyi Biotec, Bergisch-Gladbach, Germany), according to the manufacturer's protocols, and were counted as viable cells (trypan blue

Table 1. Severity of Insulinitis and Frequency of Diabetes in NOD Mice Treated with Aerosol Protein

Protein	Insulinitis score	Diabetes frequency
Insulin	1.2 ± 0.98*	2/32 (6.3%)‡
OVA	2.6 ± 0.92	8/32 (25%)

Mice (32 per group) were given either aerosol insulin or OVA for 10 consecutive days and then weekly from 28 d of age. At 105 d of age, five nondiabetic mice from each group were killed for pancreas histology. The insulinitis score is expressed as mean ± SD.

*The insulinitis score in insulin-treated mice was significantly reduced ($P < 0.01$, Mann-Whitney U test).

‡The diabetes frequency in insulin-treated mice significantly reduced ($P = 0.04$, Fisher's exact test).

stain negative). Flow cytometry revealed >95% depletion of CD4 or CD8 cells, with recoveries of ~80 and ~50%, respectively.

$\gamma\delta$ T cells were positively selected/depleted by incubating T cells from aerosol-treated mice first with biotinylated GL3-1A antibody (PharMingen) and then with streptavidin-MACS MicroBeads, followed by magnetic separation. By flow cytometry, $\gamma\delta$ cells were comprised 1–2% of NOD splenic T cells and were totally depleted with GL3-1A antibody. To purify CD8 $\gamma\delta$ T cells, CD8 T cells were first magnetically selected from total T cells with anti-CD8-FITC conjugate and anti-FITC MicroBeads. The MicroBeads were then released according to the Miltenyi Biotec protocol, and the CD8 cells were magnetically separated into $\gamma\delta$ -positive and -depleted fractions. Double staining and FACS[®] analysis (Becton Dickinson & Co., Mountain View, CA) demonstrated total depletion of $\gamma\delta$ cells and their recovery as a GL3-1A high and low expressing CD8 population.

Results and Discussion

Diabetes and Insulinitis. Aerosol human insulin or OVA was administered in different schedules to female NOD mice from 28 d of age, the earliest time at which insulinitis is detectable in our colony, and their incidence of diabetes and severity of insulinitis were subsequently measured.

The incidence of diabetes was only marginally affected by a single aerosol insulin treatment at 28 d of age, being 72% by 240 d of age, compared to 88% after aerosol OVA. However, treatment for 3 or 10 consecutive days and then weekly significantly delayed the onset and reduced the incidence of diabetes. In five separate experiments, diabetes incidence at 156 d of age was reduced from a median of 47% in OVA-treated mice to 23% in insulin-treated mice; at 240 d of age, when the cumulative incidence of diabetes approaches a maximum, the values were 79 and 49%, respectively ($P = 0.005$, Kaplan-Meier survival statistic). There was no difference if the initial treatment was for 3 or 10 d. In another experiment, in which treatment was given for 10 consecutive days and then weekly, but not started until 49 d of age, when insulinitis was well established, aerosol insulin still significantly reduced diabetes incidence at 156 d

Table 2. Proliferative Responses of Splenocytes from Aerosol-treated Mice

Additive	³ H]Thymidine uptake (cpm, mean ± SD)	
	Insulin aerosol	OVA aerosol
None	157 ± 28*	208 ± 29
Human insulin (40 µg/ml)	134 ± 27	197 ± 82
Mouse insulin II B chain (amino acids 9–23) (40 µg/ml)	169 ± 80 [‡]	435 ± 240 [¶]
Human GAD65 (20 µg/ml)	424 ± 165 [§]	1,381 ± 650 ^{**}
Con A (5 µg/ml)	3,357 ± 812	2,960 ± 494
Anti-CD3 antibody (10 µg/ml)	2,221 ± 533	2,643 ± 1,126

Splenocytes from three mice per group were assayed in quadruplicate in HL-1 serum-free medium. Statistical comparisons (Mann-Whitney U tests) were between the 12 results for each group.

§ vs * ($P = 0.001$), ¶ vs ^{||} ($P = 0.016$), ** vs ^{||} ($P = 0.001$), ¶ vs [‡] ($P = 0.002$), ** vs [§] ($P < 0.001$).

from 58 to 25% ($P = 0.001$). Insulin treatment was associated with a significant reduction in the severity of the islet lesion, as judged by the “insulinitis score,” which paralleled the decrease in diabetes incidence (Table 1). Infiltration of the salivary glands by lymphoid cells (sialitis), which also occurs in NOD mice, was unaffected by aerosol insulin.

In the absence of absorption-promoting agents, systemic uptake of insulin from the nasopharyngeal mucosa in humans is insignificant (30). In NOD mice, blood glucose was not altered in the short-term by aerosol insulin (data not shown). Insulin solutions labeled with 10% Evan's blue dye were observed to be deposited in the nasopharynx, trachea, and main bronchial divisions, as well as in the esophagus. While it may be difficult, if not impossible, to avoid some gastrointestinal exposure after aerosol or intranasal delivery of soluble protein, delivery into the nasopharynx alone is sufficient to induce tolerance (18, 31, 32).

Table 3. Cytokine Secretion by Splenocytes from Aerosol-treated Mice to 40 µg/ml of Mouse Insulin II B Chain Peptide (Amino Acids 9–23)

Cytokine	Insulin aerosol	OVA aerosol
(pg/ml)		
IL-2	200 ± 15	186 ± 27
IFN- γ	368 ± 71	423 ± 31
IL-4	36 ± 6	Not detectable
IL-10	222 ± 149	Not detectable
TGF- β	293 ± 131	162 ± 41

Supernatants from the replicate culture wells (Table 2) were sampled after a 3-d incubation and were assayed for cytokines as described in Materials and Methods.

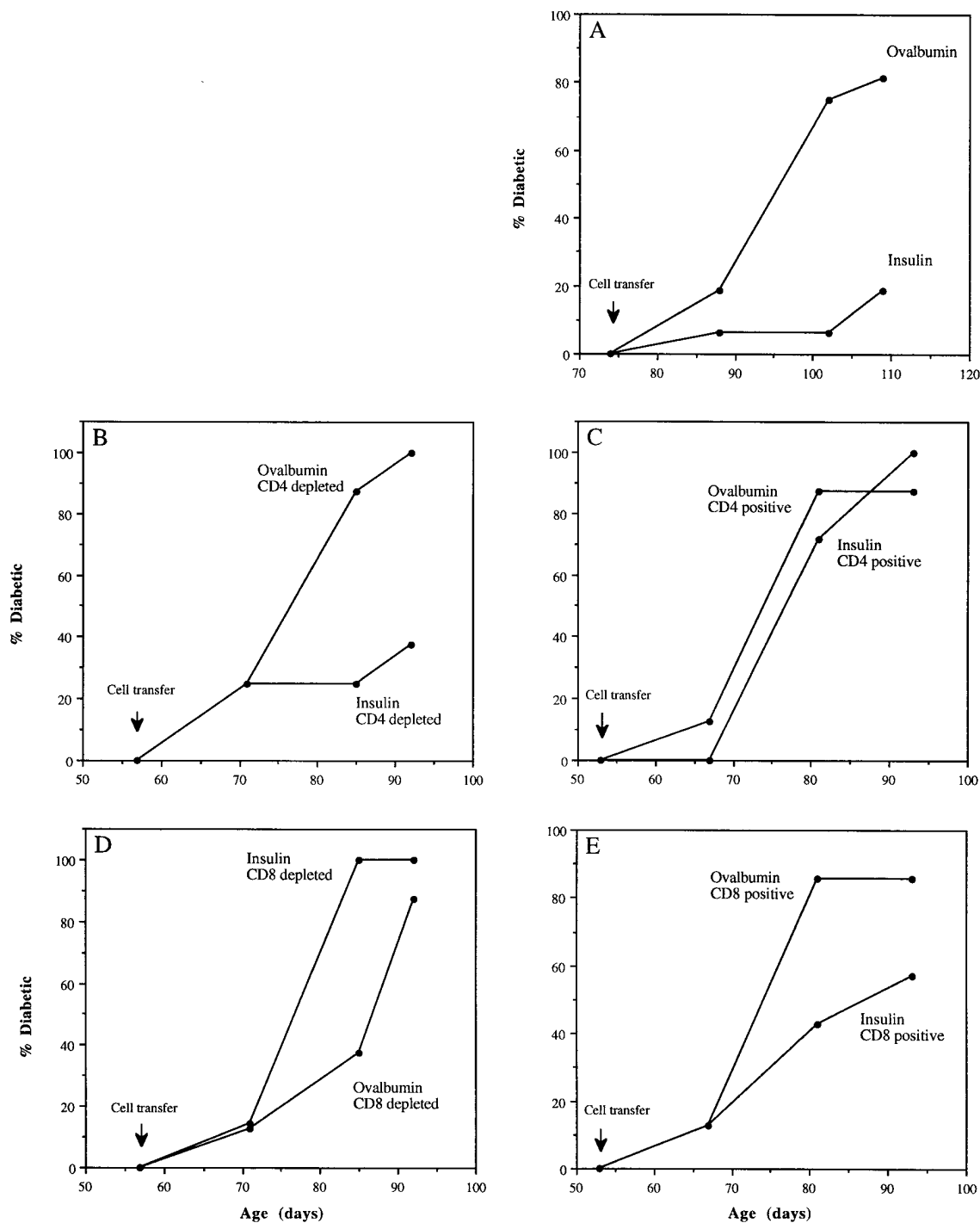


Figure 1. Aerosol insulin induces CD8 T cells that suppress the transfer of diabetes. 6–9-wk-old NOD male mice ($n = 16$ per group) were injected with pooled splenocytes from recently diabetic 14–19-wk-old females, together with either unfractated (A) or fractionated (B–E) splenocytes from aerosol insulin- or OVA-treated NOD females, and their incidence of diabetes was subsequently monitored. In the experiment shown, aerosol donor mice had been treated for 10 consecutive days and then weekly from 49 d of age, and were normoglycemic when killed at 156 d of age.

Immune Responses. We investigated if aerosol insulin treatment had altered immune responses to insulin. Unprimed T cell proliferative responses to islet antigens, including insulin, have been reported in NOD mice (33), but have not always been reproducible (34). Proliferative responses of spleen cells ($0.5\text{--}2.5 \times 10^6/\text{ml}$) from either insu-

lin- or OVA-treated mice, 56–105 d old, to human insulin or OVA (0.2, 2.0, 20, and 40 $\mu\text{g}/\text{ml}$), in different serum-supplemented or serum-free media varied by less than two-fold above basal and were usually depressed below basal at the highest concentration of insulin. Insulin at high concentrations has been reported to inhibit T cell responses

(35). In contrast, in the OVA-treated control mice, but not the insulin-treated mice, responses to insulin B chain peptide amino acids 9–23, a dominant epitope for NOD mouse islet-derived T cell clones (15), were significant (Table 2). Furthermore, OVA mice had significantly higher responses than insulin mice to human GAD65, which was previously reported to stimulate splenic T cells in NOD mice (33). In mice from both treatment groups, proliferative responses to non-antigen-specific stimulation by Con A or the T cell receptor CD3 mAb, 145–2C11 were similar (Table 2) and no different to untreated mice (not shown), indicating that aerosol treatment did not cause general immunosuppression. IL-2, IFN- γ , and TGF- β 1 secretion in response to insulin B chain amino acids 9–23 were not significantly different between insulin- and OVA-treated mice; however, the levels of IL-4 and particularly IL-10 were higher in the wells from insulin-treated mice (Table 3).

Insulin antibodies were measured by a standard immunoprecipitation assay with sera ($n = 12$ per group) from insulin- and OVA-treated mice aged 70–105 d. Precipitation of ^{125}I -insulin radioactivity by antibodies in sera from insulin-treated mice ($12.7 \pm 3.6\%$; mean precipitated cpm \pm SD) was significantly higher ($P < 0.01$, Mann-Whitney U test) than in OVA-treated mice ($6.9 \pm 2.6\%$). This increase in the “level” of insulin antibodies after aerosol insulin, together with the suppression of T cell proliferation and the increase in IL-4 and IL-10 responses to the insulin B chain peptide, is consistent with the phenomenon of immune deviation, as described after oral myelin basic protein in Lewis rats (16) and intranasal GAD peptides in NOD mice (22). β cell destruction within the DTH lesion of IDDM is an example of a Th1-mediated process (1, 2), whose inhibition by aerosol insulin might be expected to shift the Th1/Th2 balance towards Th2 in response to key islet antigens. Defective suppressor T cell function has been postulated to shift the balance towards Th1 in IDDM (2). It seems unlikely that the reduced T cell proliferative response to GAD could reflect “bystander” suppression caused by the secretion of the Th2 cytokines IL-4 and IL-10 (16) by insulin aerosol-induced regulatory cells because, apart from an absence of added insulin in the cultures with GAD, responses to Con A and anti-CD3 were not impaired. A direct explanation is that the reduced response to GAD reflects the protective effect of aerosol insulin on insulinitis and β cell destruction. This implies that at least some GAD immunity is secondary, and that immunity to (pro)insulin may have a more proximal role in β cell destruction. Although NOD mouse T cell responses to human GAD65 have been reported to be stronger and to appear earlier than those to native human insulin (33), we have recently shown (36) that the transgenic expression of mouse proinsulin II in NOD mouse APCs completely prevents insulinitis and diabetes.

Regulatory CD8 $\gamma\delta$ T Cells. As mucosal tolerance has been associated with the appearance of “regulatory” cells in the spleen (16, 18, 20–23), we asked if aerosol insulin-induced regulatory cells that could inhibit the adoptive transfer of diabetes by pathogenic effector T cells. In the

classic adoptive transfer model (37; see Fig. 3), spleen cells from diabetic NOD female mice transferred intravenously to young, irradiated, nondiabetic, syngenic male or female recipients cause clinical diabetes in the majority within 4 wk. When we coinjected 2×10^7 spleen cells from older, diabetic mice with an equal number of spleen cells from aerosol OVA mice, the majority of young recipients developed diabetes within 4–5 wk; in contrast, after coinjection with spleen cells from aerosol insulin mice, only a minority developed diabetes (Fig. 1 *A*). Diabetes incidence was suppressed by $\geq 75\%$ in six separate experiments with either splenocytes or nylon wool-nonadherent splenocytes (enriched for T cells) from aerosol insulin mice.

Spleen cells were then fractionated to identify the regulatory cells responsible for the suppression of diabetes transfer. Depletion and positive selection of CD4 and CD8 cells clearly showed that CD8 cells were wholly responsible for the suppression of transfer (Fig. 1, *B–E*). Depletion of CD4 cells did not alter the ability of residual spleen cells from aerosol insulin mice to suppress transfer (Fig. 1 *B*), and positively selected CD4 cells did not suppress transfer (Fig. 1 *C*). On the other hand, there was no suppression by CD8-depleted spleen cells from aerosol insulin mice (Fig. 1 *D*), whereas positively selected CD8 cells suppressed transfer (Fig. 1 *E*). The partial suppression by positively selected CD8 cells, in contrast to the rapid development of diabetes after their depletion, is probably caused by inefficient recovery of CD8 cells; in this experiment, 7×10^5 purified CD8 cells were coinjected into each recipient with 2×10^7 spleen cells from diabetic mice.

T cells bearing $\gamma\delta$ receptors have been shown to have an immunoregulatory role (24–29). Interestingly, it has been reported that total peripheral blood $\gamma\delta$ cells decrease concomitantly with the loss of β cell function in humans with subclinical IDDM (38). McMenamin et al. (23) found that small numbers of $\gamma\delta$ T cells could adoptively transfer the suppression of OVA-specific IgE responses induced in mice by repeated inhalation of OVA. To determine if the suppression of diabetes transfer that we observed was caused by $\gamma\delta$ T cells, we fractionated spleen cells with the anti- $\gamma\delta$ T cell mAb GL3-1A (39). Depletion of $\gamma\delta$ T cells, like that of CD8 cells, completely abrogated the ability of nylon wool-nonadherent spleen cells from insulin aerosol-treated mice to suppress the adoptive transfer of diabetes (Fig. 2 *A*). Conversely, relatively small numbers of $\gamma\delta$ T cells from insulin aerosol-treated mice could suppress transfer. Diabetes incidence after transfer was decreased by 50% for at least 70 d when 1.4×10^5 $\gamma\delta$ T cells were coinjected with 2×10^7 spleen cells from diabetic mice (Fig. 2 *A*). The splenic CD8 and $\gamma\delta$ T cells that suppressed diabetes transfer were one and the same, and not two interdependent populations. Thus, the ability of CD8 cells from insulin aerosol-treated mice to suppress transfer was abolished if they were first depleted of $\gamma\delta$ T cells, whereas small numbers of $\gamma\delta$ cells purified from the CD8 cells prevented transfer (Fig. 2 *B*). A summary of the results from 11 different cotransfer experiments is presented in Fig 3.

FACS[®] analysis revealed that $\gamma\delta$ cells reactive with GL3

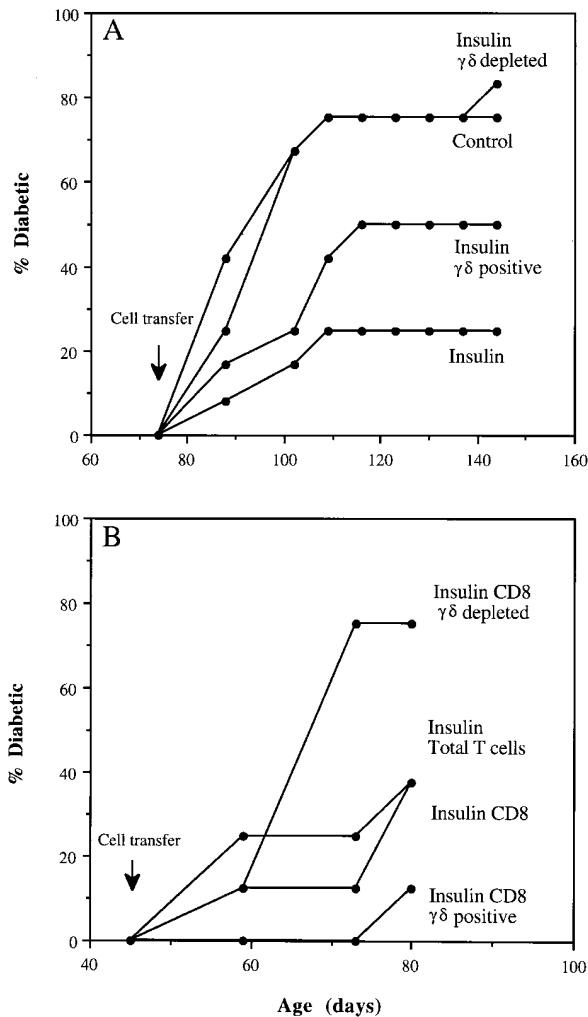


Figure 2. Aerosol insulin induces CD8 $\gamma\delta$ T cells that suppress transfer of diabetes. Young male NOD mice were coinjected with “diabetic” splenocytes (2×10^7) and total or fractionated splenic T cells from aerosol-treated mice, as described in the legend to Fig. 1. The numbers of fractionated cells injected were, (A) $\sim 10^7$ total T cells and, from aerosol insulin mice, $\sim 10^7$ $\gamma\delta$ -depleted T cells or 1.4×10^5 $\gamma\delta$ T cells and, (B) from aerosol insulin mice, $\sim 10^7$ total T cells, 2×10^6 CD8 T cells, 2×10^6 $\gamma\delta$ -depleted CD8 T cells, or 1.5×10^5 CD8 $\gamma\delta^+$ T cells.

antibody constitute 1.6–2.4% of total and $\sim 1\%$ of CD8⁺ T cells in the spleens of 12–16-wk-old female NOD mice. These values were no different between groups of mice treated with insulin or OVA aerosol. However, because of their low abundance distinct subpopulations of antigen-specific CD8 $\gamma\delta$ T cells would be difficult to distinguish this way. The higher protection with fractionated cells, e.g., sequentially purified CD8 $\gamma\delta$ cells (Fig. 3), is quantitative and reflects their higher absolute number relative to that in unfractionated cells.

Aerosol inhalation as a mode of insulin delivery to the mucosa was as effective as oral insulin (20, 21) in reducing diabetes incidence in the NOD mouse. The fact that it was therapeutic after the onset of insulinitis is especially relevant to the prevention of IDDM in at-risk humans with subclinical disease in whom the presence of circulating islet antigen-

CD8 $\gamma\delta$ T cells from insulin aerosol-treated mice block adoptive transfer of diabetes

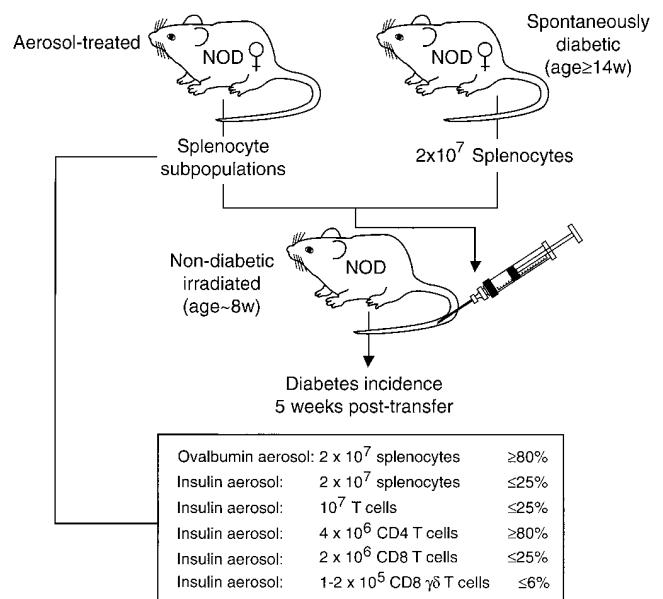


Figure 3. Adoptive transfer of diabetes is suppressed by CD8 $\gamma\delta$ T cells induced by aerosol insulin: summary of 11 experiments.

reactive antibodies and T cells is taken to reflect underlying insulinitis. Indeed, compared to humans with recently diagnosed IDDM, NOD mice have more intense insulinitis and the majority of females progress to diabetes (1, 2, 4). Aerosol insulin had no obvious metabolic effect, but it induced a population of regulatory CD8 $\gamma\delta$ T cells, small numbers of which suppressed the ability of pathogenic effector T cells to adoptively transfer diabetes. These antigen-induced “suppressor” T cells that are protective against cell-mediated autoimmune pathology have not been described previously. The striking potency of these cells, unparalleled in “infectious suppression,” was noted by McMenamin et al. (23) in their experimental model of OVA aerosol-induced suppression of OVA-specific IgE and T cell responses, and implies they have the capacity to expand *in vivo* after transfer. The ability of CD8 $\gamma\delta$ T cells to suppress, on the one hand, cell-mediated autoimmune disease in NOD mice and, on the other, an “allergic” response to exogenous OVA in C57Bl mice (23) or Brown Norway rats (40) seems paradoxical within the framework of the Th1–Th2 paradigm. However, the overriding consideration is that mucosa-mediated tolerance is manifest in genetically susceptible strains by suppression of both DTH and IgE responses (18); either the strict dichotomy of the Th1–Th2 paradigm may not apply and/or there may be rodent strain variations in mucosa-mediated tolerance responses that reflect differences in Th1–Th2 balance. It would be interesting to determine if OVA aerosol induces CD8 $\gamma\delta$ T cells that suppress OVA-specific IgE responses in NOD mice.

Oral tolerance has been associated with a decrease in cellular and sometimes an increase in humoral antigen-specific

immunity, and with either CD8 or CD4 T cells that secrete, respectively, TGF- β or IL-4, IL-10, and TGF- β 1 (16). However, these regulatory cells have not been identified as bearing $\gamma\delta$ receptors. In NOD mice, oral tolerance to insulin was attributed to regulatory CD4 T cells (21). We have shown that CD8 $\gamma\delta$ T cells account entirely for the regulatory cells induced by aerosol insulin. This finding raises the possibility that the mechanisms and regulatory cells that mediate mucosal tolerance in the upper airways and gut are different. Experiments are in progress to identify the origin, fate, antigen specificity, and exact mode of action of insulin aerosol-induced regulatory CD8 $\gamma\delta$ T cells.

Total $\gamma\delta$ T cells in the spleens of NOD female mice de-

crease with age similarly to BALB/c mice (41), but the role of specific $\gamma\delta$ T cells in the natural history of diabetes in the NOD mouse remains to be defined. In subclinical IDDM subjects, a decrease in the number of peripheral blood V γ 9V δ 2 T cells signaled loss of β cell function and progression to clinical diabetes (38). We suggest, therefore, that peripheral regulatory CD8 $\gamma\delta$ T cells could have a critical role in determining the natural history of islet autoimmunity. Moreover, the induction of these regulatory cells by aerosol delivery of insulin (or other islet antigens) has implications for the prevention of clinical diabetes in at-risk humans.

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