Rapid communications

Insulin selectively primes Th2 responses and induces regulatory tolerance to insulin in pre-diabetic mice

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Summary Little is known about the immunological impact of insulin administration other than it can boost insulin autoantibody levels. In particular, while the subcutaneous administration of a soluble foreign antigen (without adjuvant) is generally only weakly immunogenic in a naive animal, it is unknown what effect the subcutaneous administration of a soluble self-antigen has in animals with established autoimmune responses to the antigen. Addressing these questions in pre-diabetic nonobese diabetic (NOD) mice, we examined the effects of administering insulin, as well as the metabolically inactive B-chain of insulin, on insulin-specific cellular and humoral immune responses. We show that pre-diabetic NOD mice have a spontaneous Th1-biased response against insulin. Administering insulin, or the insulin B-chain, rather than boosting the established Th1 response, primed Th2 cellular and humoral immunity to insulin, shifting the predominant insulin response toward a Th2 phenotype. Despite the presence of a Th1 response against insulin, insulin treated mice failed to mount proliferative T-cell responses following immunization and challenge with insulin, demonstrating that the treatment induced an active form of tolerance to this autoantigen. Thus, the subcutaneous administration of a soluble antigen can engage Th2 responses and induce self-tolerance, even after the establishment of autoreactive Th-1 responses. Such immune deviation and induced regulatory tolerance may contribute to the protective effects of prophylactic insulin therapy, as well as the establishment of a "honeymoon" phase in new-onset insulin-dependent diabetic patients. [Diabetologia (1998) 41: 237–240]

Keywords Th2, Th1, immunotherapy, autoimmunity, NOD, insulin, diabetes, tolerance, self-antigen.

There is conflicting information as to the existence, prevalence and phenotype of cellular immune responses to insulin in pre-diabetic rodents and humans [1–6]. Furthermore, little is known about the immunological impact of insulin administration, other than it can boost insulin autoantibody levels ([7] and references therein). The subcutaneous administration of soluble antigen has been traditionally thought to be only weakly immunogenic in naive animals, necessitating the use of adjuvants to prime significant immune responses. However, it has recently been shown that the subcutaneous administration of prototypic soluble foreign antigens can prime naive T cells toward a Th2 phenotype [8]. This finding raises the question whether the subcutaneous administration of self-antigens, such as insulin to pre-diabetic and diabetic individuals, also induces Th2 responses. If insulin administration does have an immunological impact, the nature of this response (Th1/Th2) may depend on whether an autoimmune response to insulin is already established and the phenotype of this response. If a memory Th1 response to insulin is present in pre-diabetic and diabetic animals, insulin injection may boost this pro-inflammatory response. On the other hand, if insulin adminis-
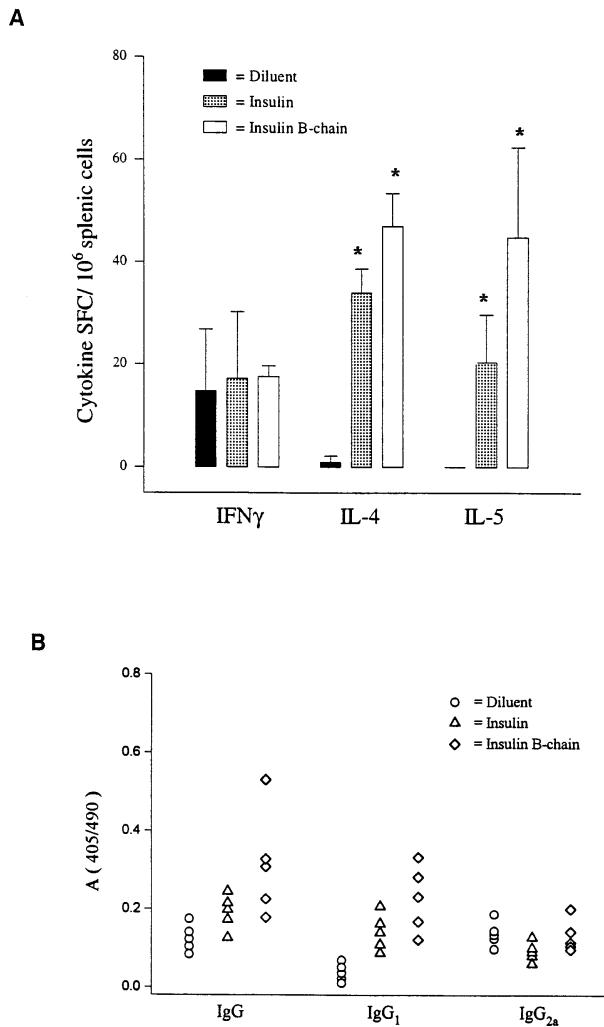
A

B

Fig. 1. A) Insulin and insulin B-chain administration induce Th2 responses. Splenic T cells from diluent (■), insulin (▦), or insulin B-chain (□) treated NOD mice were isolated and the frequency of insulin-specific T cells secreting IFN γ, IL-4 and IL-5 was determined by ELISPOT. The data are represented as the mean number of spot forming colonies (SFC) per 10^6 splenic T cells ± SEM. The background level was ≤ 5 SFC. Experimental and control mice were tested simultaneously (in triplicate) in two separate experiments ($n = 5$ for each group). * Response compared to diluent control $p < 0.001$. No responses were detected to control HEL_{11-25} peptide (data not shown). Responses by splenic T cells from untreated BALB/c mice were at background levels (data not shown). **B)** Both insulin and insulin B-chain administration boost IgG_1 antibody responses to insulin. Insulin antibodies in sera from diluent (○), insulin (△) and insulin B-chain (◊) were characterized using subclass-specific ELISA assays. Serial dilutions of sera showed a linear relationship with resulting optical density (OD). The data are represented as the mean absorbance values over background of triplicate samples from individual mice ($n = 5$ for each group). The background OD was approximately 0.05 ± 0.01 for all samples. Experimental and control sera were tested simultaneously in two separate assays. Antibodies to insulin in sera from untreated BALB/c and AKR mice were at background levels (data not shown). For total IgG, $p < 0.04$ for insulin and < 0.01 for insulin B-chain relative to control diluent treated mice. For IgG1 $p < 0.02$ for insulin and < 0.005 for insulin B-chain relative to diluent treated mice. There was no significant difference in IgG_{2a} levels between the groups period.

tration induces Th2 responses de novo, or boosts established Th2 responses, these cells could promote beta cell survival by virtue of secreting anti-inflammatory cytokines in the microenvironment of the islets ([9] and references therein). Alternatively, determinants of the exogenous insulin could also be displayed by non-professional antigen presenting cells (APC), leading to the inactivation of antigen-specific T cells.

Using a high resolution ELISPOT assay we examined the nature of the spontaneous cellular autoimmune response to insulin and the immunological impact of administering insulin, as well as the metabolically inactive insulin B-chain (which contains insulin's dominant determinant ([6] and references therein) in pre-diabetic NOD mice.

Materials and methods

Eight-week-old female NOD mice (Taconic Farms, Germantown, NY) mice received s.c. 0.1 ml containing 50 µg insulin (Humulin U; Lilly, Indianapolis, IN) in diluent (a dose similar to that given to diabetic NOD mice), 50 µg bovine insulin B-chain (Sigma, St. Louis, MO) in diluent, or diluent alone. This treatment was repeated every other day 9 more times. Ten days following the last treatment, the frequency of splenic insulin-specific T cells secreting interferon γ (IFN γ), interleukin-4 (IL-4), and IL-5 was determined using an ELISPOT technique ([9] and references therein). Insulin B-chain or a control hen eggwhite lysozyme peptide (HEL_{11-25}, which contains a dominant determinant) was present at 20 µmol/l. Insulin autoantibodies were characterized by an ELISA assay as previously described [9], with the exception that insulin B-chain (10 µg/ml) was bound to the wells.

Other groups of diluent, insulin or insulin B-chain-treated NOD mice were immunized (10 days following their last treatment as described above) with insulin B-chain (100 µg) in 50 % complete Freund's adjuvant (CFA) in the hind footpad. Nine days later, splenic (8×10^5) and draining lymph node (4×10^5) T cells were challenged with different dosages of insulin B-chain in vitro and the proliferative responses were determined as previously described [9].

Results and Discussion

Insulin and insulin B-chain administration, rather than boosting an established Th1 response, prime Th2 responses to insulin. We administered insulin, the metabolically inactive insulin B-chain (in diluent), or diluent alone, subcutaneously to 8-week-old pre-diabetic NOD mice and 30 days following the initiation of treatment we measured the frequency of splenic Th1 and Th2 insulin-specific T cells directly ex vivo by ELISPOT. In control-diluent treated mice, we readily detected IFN γ secreting insulin-specific splenic Th1 cells at a frequency of about one per 67,000 T cells (Fig. 1 A). The frequency of insulin-reactive Th1 cells was not significantly different in mice that had been treated with insulin or the insulin B-chain ($p > 0.05$).
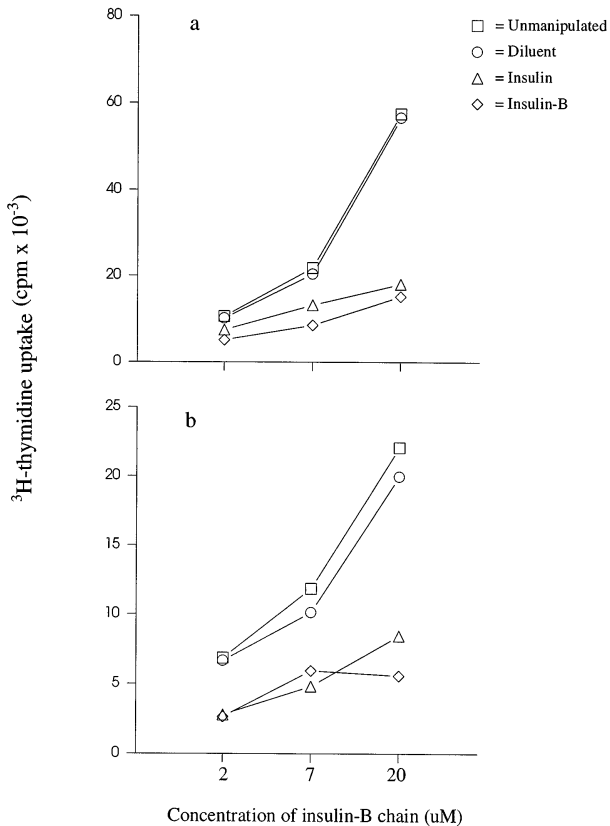
Fig. 2 a, b. Administration of insulin or insulin B-chain inhibits proliferative recall responses to insulin. Draining lymph node (**a**) and splenic (**b**) T cells from unmanipulated (□), diluent (○), insulin (△) and insulin B-chain (◇) treated mice were tested for proliferative responses to insulin B-chain in vitro. Responses to control PPD (10 μg/ml) were similar in both experimental and control groups. Background for medium alone ranged from 1,800–4,500 cpm. 4–6 mice from each group were tested simultaneously in two separate experiments (using triplicate cultures)

Thus, pre-diabetic NOD have a spontaneous insulin-specific Th1 response and the administration of insulin or it's B-chain did not appear to have a significant impact on this component.

In control diluent treated mice, the frequency of insulin-specific IL-4 secreting T cells was at background levels (Fig. 1 A). Thus, the spontaneous proliferative insulin-specific T-cell response we previously described in pre-diabetic NOD mice [4] is highly Th1-biased. Following the administration of insulin or the insulin B-chain, the frequency of IL-4 secreting insulin-specific T cells rose 34 and 47-fold (i.e. to 1 per 29,000 and 1 per 21,000 T cells), respectively (Fig. 1 A). We observed a similar increase in IL-5 secreting insulin-specific T cells, another Th2 cell cytokine (Fig. 1 A). Indeed, following insulin and insulin B-chain treatment, insulin-specific splenic Th2 cells became on average, more frequent than Th1 cells. These data demonstrate that rather than boosting the established Th1 response, both insulin and insulin B-chain administration induced Th2 responses, shifting the predominant insulin response toward a Th2 phenotype.

Insulin and insulin B-chain administration induces IgG$_1$ insulin-specific antibodies. Control diluent treated pre-diabetic NOD mice had low levels of insulin IgG antibodies (Fig. 1 B). Mice treated with insulin or insulin B-chain had higher levels of insulin-reactive IgG antibodies which were predominantly of the IgG$_1$ subclass, indicating the priming of insulin-specific Th2 help. Notably, high autoantibody titres have been associated with slower insulin-dependent diabetes progression in both NOD mice and humans ([9] and references therein). In contrast, the level of IgG$_{2a}$ insulin autoantibodies, which reflect Th1 help, remained at low levels in both experimental and control groups. These data confirm our observations using ELISPOT that insulin and insulin B-chain administration did not promote the established Th1 response, but rather primed an insulin-specific Th2 response.

Treatment with soluble insulin or insulin B-chain induces regulatory tolerance. To examine the functional significance of the observed shift in insulin-specific Th1/Th2 responses, we examined the ability of experimental and control mice to mount recall responses to insulin. After treatment with diluent, insulin or insulin B-chain, mice were immunized with insulin B-chain in CFA and tested for proliferative T cell responses to insulin B-chain in vitro. Both splenic and lymph node T cells from diluent-treated animals displayed strong proliferative responses to insulin B-chain, at levels similar to those observed in unmanipulated pre-diabetic NOD mice (Fig. 2). In contrast, splenic and lymph node T-cell responses to insulin were significantly reduced, or almost abolished, in mice treated with insulin or insulin B-chain ($p < 0.03$ or $p < 0.001$ for 7 and 20 μmol/l insulin B-chain, respectively). As ELISPOT analysis revealed that insulin-reactive Th1 cells are still present (Fig. 1 A), these data suggest that the primed insulin-specific Th2 cells actively down-regulated Th1 proliferation in vitro. Thus, even after the establishment of autoreactive Th1 responses, the administration of autoantigen (independent of adjuvants) can engage Th2 responses and induce regulatory tolerance.

Guéry et al. [8] recently reported that administration of soluble prototypic foreign antigens can prime Th2 responses in naive animals. Our data extend these findings by demonstrating that soluble self-antigens can engage Th2 responses in the presence of an established Th1 autoimmune response–allowing that the antigens used in our study be considered self-antigens since they differ from their murine counterparts by only a one amino acid conservative substitution in a region which does not comprise a T-cell determinant ([6] and references therein). While these data

suggest that the administration of any soluble determinant via the subcutaneous route will tend to elicit Th2 responses, the ability of a self-antigen to do this in the presence of primed Th1 responses to the self-antigen is significant, as it may direct anti-inflammatory responses to a target tissue.

We did not observe a significant change in the frequency of insulin-reactive Th1 cells in mice treated with insulin, suggesting that if a reduction in the presentation of beta-cell autoantigen determinants occurred through "beta-cell rest", it was not sufficient to impact the insulin-specific Th1 component over the course of this study. The apparent lack of impact on the Th1 component also suggests that the deletion/inactivation of insulin-specific T cells due to the presentation of insulin determinants by non-professional APC, is not a frequent outcome under the conditions used in this study. Finally, our observation that the insulin B-chain primes Th2 responses to the same (or greater) degree as intact insulin argues against the hypothesis that insulin promotes Th2 responses through binding to the insulin receptor on T cells and inducing a signalling pathway that shares elements with the IL-4 signal transduction pathway [10].

In conclusion, we have shown that even in the presence of an established Th1 autoimmune response, insulin treatment can prime antigen-specific Th2 responses and promote regulatory tolerance to this autoantigen. This induction of Th2 immunity may account for the high percentage of individuals who develop insulin autoantibodies following insulin therapy ([7] and references therein). Numerous studies in animal models of autoimmune disease suggest that the induction of Th2 responses to autoantigens may down-regulate pathogenic Th1 responses in a target tissue. If insulin treatment induces a similar immune deviation process in humans, it may contribute to the protective effects of prophylactic insulin therapy, as well as the establishment of the "honeymoon" phase in new-onset insulin-dependent diabetic patients.

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