Changes in function of antigen-specific lymphocytes correlating with progression towards diabetes in a transgenic model

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Mice that express influenza hemagglutinin under control of the rat insulin promoter (INS-HA) as well as a class II major histocompatibility complex (MHC)-restricted HA-specific transgenic TCR (TCR-HA), develop early insulinitis with huge infiltrates, but progress late and irregularly to diabetes. Initially, in these mice, INS-HA modulates the reactivity of antigen-specific lymphocytes, such that outside the pancreas they do not cause lethal shock like their naive counterparts in single transgenic TCR-HA mice, when stimulated with high doses of antigen. Inside the pancreas, the antigen-specific cells do not initially attack the islet cells, and produce some IFN- γ as well as IL-10 and IL-4. Spontaneous progression to diabetes, which can be accelerated by cyclophosphamide injection, is accompanied by a 10-fold increase in IFN- γ and a 3-fold decrease in IL-10 and IL-4 production by the locally residing antigen-specific T cells. Also, total islets from non-diabetic mice contain more TNF- α , compared with diabetic mice. This scenario is consistent with the view that β cell destruction depends upon the increased production of certain pro-inflammatory cytokines by infiltrating T cells. Our inability to detect Fas expression on β cells, but not on lymphoid cells, in diabetic and non-diabetic mice, puts some constraints on the role of Fas in β cell destruction.

Keywords: transgenic mice/cytokine expression/diabetes

Introduction

Autoimmunity can result in tissue destruction and functional impairment caused by autoreactive lymphocytes. Even though a better understanding concerning the target antigens and genes involved in several autoimmune diseases has been accumulating during recent years, we still remain fairly ignorant of the initial events leading to autoimmune aggression. Experimental models of autoimmune disease, in which transgene-encoded antigens are expressed in specific organs, have shown that, in most of the cases, the presence of self-reactive T cells in secondary lymphoid organs, even at high frequency, is not in itself sufficient for the development of aggressive autoimmunity.

Therefore, there must be mechanisms that keep such self-reactive T cells in check. In cases where the expression of a given antigen has been mostly restricted to the pancreatic β cells, different outcomes have been observed, probably depending on the nature of the antigen, the frequency of reactive cells and the affinity of the reactive cell's T cell receptor (TCR). Antigen was apparently ignored upon expression of the lymphocytic choriomeningitis viral (LCMV) glycoprotein (GP-33) in islets, even when the number of self-reactive cells was high, due to the introduction of a transgenic, class I major histocompatibility complex (MHC)-restricted, GP-33 specific TCR (Ohashi *et al.*, 1991). These mice developed insulinitis and diabetes only upon infection with virus. Ignorance was also observed when mice expressing the MHC class I molecule K^b, under the control of the rat insulin promoter (Rip-K^b), were crossed to mice transgenic for a K^b-specific TCR: these mice were capable of rejecting K^b skin grafts, but did not develop insulinitis or diabetes thereafter (Heath *et al.*, 1992). These situations may represent exceptional cases where class I MHC molecules, or peptides bound to class I MHC molecules, are not at all, or only poorly presented by antigen-presenting cells (APCs) neighboring the β islet cells, and therefore do not activate T cells. With regard to other class I, and especially class II MHC-presented peptides, one might assume that antigens expressed by the β cells will, as a rule, be presented by local professional APCs. Thus, immunological ignorance may represent an exception to the rule.

In cases where β cell antigens are presented by local APCs, the frequency of reactive cells appears to represent an important parameter in deciding whether or not diabetes develops. In mice expressing the simian virus 40 large T antigen (Tag) under the control of the insulin promoter, tolerance by deletion and anergy of MHC class II-restricted Tag-TCR cells was observed when their frequency was low (10%), but massive insulinitis was observed in mice where the frequency of Tag-TCR cells was high (90%) (Förster *et al.*, 1995). Non-MHC genes may also play a role: in mice expressing the hemagglutinin (HA) of the influenza virus under the insulin promoter (INS-HA), crossed onto mice transgenic for a HA-specific TCR restricted to I-A^d, very mild insulinitis was observed in BALB/c mice, while aggressive insulinitis developed on the B10.D2 background (Scott *et al.*, 1994). This could have been due to the different cytokine secretion profile of the reactive T cells in the different mouse strains, as in other experiments it was shown that T-helper 1 (Th1) but not T-helper 2 (Th2) CD4⁺ T cells, secreting different cytokines, caused diabetes (Katz *et al.*, 1995). Finally, the affinity of the auto-reactive TCR may also be important: the same INS-HA mice developed massive insulinitis and overt diabetes when crossed to mice transgenic for another anti-HA TCR (TCR-HA), restricted to I-E^d, regardless of the genetic background (Degermann *et al.*, 1994).

In the TCR-HA×INS-HA double transgenic model described here, the transgenic TCR is expressed at high levels on ~10% of peripheral CD4⁺ and CD8⁺ T cells that are neither ignorant of, nor tolerant to, the HA antigen on pancreatic tissue, because massive insulinitis is observed at an early age in these animals, and because T cells from spleen and lymph nodes proliferate similarly to T cells from single TCR-HA transgenic mice when stimulated by HA. In the past, these studies have led to the conclusion that the reactive T cells in peripheral lymphoid tissue are not measurably influenced by the antigens in the pancreas (Degermann *et al.*, 1994; Scott *et al.*, 1994). We have re-examined these questions by analyzing the *in vivo* reactivity of cells with the transgenic TCR, in single and double transgenic mice. In addition, in a first attempt to dissect events that are responsible for the transition from insulinitis to diabetes, we have analyzed the cellular composition, as well as the cytokine production, in mice with either insulinitis or insulinitis and diabetes. For this, the TCR-HA×INS-HA model appears to be particularly suited because of the availability of a monoclonal antibody that permits one to address the role of antigen-specific T cells in the disease process.

Our results show clear-cut differences in reactivity and effector functions of the antigen-specific T cells which correlate with exposure to antigen and progression to disease.

Results

Encounter with HA antigen in INS-HA mice changes reactivity of T cells in secondary lymphoid organs

As previously described (Degermann *et al.*, 1994), the presence of the HA antigen in the pancreas did not significantly affect the absolute numbers of 6.5⁺ cells present in primary and secondary lymphoid organs of double transgenic mice, as compared with single TCR transgenic mice. Furthermore, the proliferative capacity of peripheral 6.5⁺ cells from double transgenic mice, upon antigenic stimulation *in vitro*, was similar to that of 6.5⁺ cells from single TCR transgenic mice. While these experiments were initially interpreted to indicate that outside of pancreatic islets, HA-specific T cells were in a similar functional state to those in single TCR-HA transgenic mice, the data in Figure 1 indicate that this population of cells behaves differently in the different types of mice: upon injection of high antigenic doses, equal to 1 mg of peptide or superior to 0.5 mg of the chimeric HA-Ig (described in Lanoue *et al.*, 1997), eight out of nine single TCR transgenic mice died ~24 h following the injection, whereas six out of seven double transgenic mice survived. Upon analysis of mice 20–24 h after antigen injection, we found that all 6.5⁺ cells from single TCR transgenic mice, but only a portion of cells from double transgenic mice, had dramatically upregulated Fas, CD25 and CD69 expression (Figure 1). CD45RB expression did not change after antigen injection and was reproducibly lower in double transgenic mice. Therefore, these results suggest that exposure of 6.5⁺ cells to antigen in TCR-HA×INS-HA mice has changed their reactivity such that they have a less aggressive phenotype and are no longer as harmful as their naive counterparts.

Fig. 1. Activation of 6.5⁺ cells in TCR-HA and TCR-HA×INS-HA mice after injection of high doses of antigen. Mice were injected i.p. with 1 mg of HA peptide and analysed 20 h after injection. Single cell suspensions were prepared from the lymph nodes and spleen of these mice and two-color staining was performed using 6.5 and Fas (CD95), CD25, CD69 or CD45RB antibodies. Histograms were obtained by gating on live, 6.5^{hi} expressing cells. The dotted line in the first histogram represents the negative control.

Predictable development of insulinitis by 2 weeks of age

As previously described (Degermann *et al.*, 1994), 6.5⁺ cells in TCR-HA×INS-HA double transgenic mice do not ignore the antigen expressed in the pancreas. On the contrary, heavy infiltrates could be observed in the islets of these mice. The kinetics of infiltration in the double transgenic mice was determined by analyzing pancreatic sections at different timepoints after birth. We found that insulinitis started at two weeks after birth, a timepoint which coincides with the maturation of the peripheral immune system and with the appearance of a significant number of 6.5⁺ cells in the spleen and lymph nodes (data not shown). By 4 weeks of age, all islets examined contained very large numbers of infiltrating lymphocytes, though islands of insulin-secreting cells remained intact (Figure 2A and B). These kinetics of infiltration were similar to, but faster than those described for another transgenic model of diabetes (Katz *et al.*, 1993). The results show that newly formed T cells are being recruited quickly by antigen expressed locally in the pancreas.

Antigen-specific T cells are enriched in pancreatic infiltrates

In order to study the phenotype of infiltrating cells in more detail, we performed immunohistochemistry on pancreatic sections of mice at 4 weeks of age, and found that the majority of infiltrating lymphocytes were CD4⁺ cells. Nevertheless, CD8⁺ cells, as well as B cells, were also present. Macrophages were less abundant and were

Fig. 2. Lymphocyte infiltration and insulin content of the pancreatic islets from an 8-week-old non-diabetic TCR-HA×INS-HA mouse (A and B), compared with a diabetic TCR-HA×INS-HA mouse from the same litter (C and D). Sections were stained with hematoxylin and eosin (A and C) or incubated with an insulin antibody, and revealed by the peroxidase substrate AEC (B and D). Red staining represents insulin-producing β cells (magnification 100 $\times$).

localized at the periphery of the islets (data not shown). The infiltrated islets in the double transgenic mice were considerably bigger in size than those observed in another model of diabetes, the non-obese diabetic (NOD) mouse.

By cytofluorometry, it was found that at 4 weeks, the percentages of CD4⁺, CD8⁺ and B220⁺ cells among infiltrating lymphocytes were similar to those found in the lymph nodes. There was, however, a significant 4- to 5-fold enrichment of both CD4⁺ and CD8⁺ antigen-specific cells (stained with the 6.5 mAb), when compared with lymph nodes (Figure 3A and Table I). These data indicate that entrance of lymphocytes to the pancreas is strongly influenced by antigen, but is not restricted exclusively to antigen-specific lymphocytes. This notion is supported by the finding that these bystander lymphocytes apparently were not recognizing, and being activated by, other pancreatic antigens to the same extent, because the antigen-specific (6.5⁺) cells showed significantly higher levels of the early activation marker, CD69, in comparison with 6.5⁻ cells present in the pancreas. Also, 6.5⁺ cells in the pancreas were more activated than 6.5⁺ cells in the lymph nodes and spleen (Figure 3B).

Progression to diabetes

Despite the fact that the pancreata of all double transgenic mice were heavily infiltrated by the age of 4 weeks, only

30–40% of these mice developed overt diabetes in our mouse colony. The age of disease onset (defined by glucose levels in blood >200 mg/dl) varied between 4 and 17 weeks of age (Figure 4). Surprisingly, once the TCR-HA×INS-HA mice became diabetic, they could survive for several weeks, contrary to what has been observed in NOD mice, where mice die within a week or two of disease onset.

We have addressed the question of whether the progression from insulinitis to diabetes was accompanied by any obvious changes in the infiltrates, by comparing the lymphocytes infiltrating the pancreas of diabetic versus non-diabetic mice. No significant difference could be observed between the degree of lymphocyte infiltration in diabetic versus non-diabetic mice, and in both cases, insulin was still detected in intact β islet cells (Figure 2A–D), explaining why these mice could survive for several weeks after disease onset. Again, no major differences could be observed either in the frequency or in the islet distribution of CD4⁺, CD8⁺, B220⁺ or Mac-1⁺ cells (not shown). When comparing activation markers, for instance CD69 and CD25, we found that HA-specific cells were significantly more activated than other lymphocytes present in the islets, but no significant difference could be observed between lymphocytes from mice with insulinitis versus lymphocytes from diabetic mice, with regard to

Fig. 3. (A) Percentage of 6.5⁺ cells infiltrating the islets of a 4-week-old TCR-HA×INS-HA mouse. Single cell suspensions, corresponding to infiltrating lymphocytes in the pancreas, or to peripheral lymphocytes of the same mouse, were used for three-color staining with CD4-PE, CD8-Red613 and 6.5-Fluos antibodies. Histograms show the percentage of 6.5⁺ cells gated for CD4 or CD8 expression. These results were reproducible. (B) Analysis of 6.5⁺ cells infiltrating the islets of a 4-week-old TCR-HA×INS-HA mouse. Two-color staining was performed on single cell suspensions with the 6.5 antibody and CD69, CD25 or CD62-L antibodies. The dotted lines on the histograms of the first row represent the negative control. Histograms were obtained by gating on 6.5⁺ or 6.5⁻ cells. Antigen-specific (6.5⁺) cells in the pancreas are compared with 6.5-negative cells in the pancreas and to 6.5-positive cells in the lymph nodes.

CD69 and CD25 activation markers (data not shown). In both types of mice, 6.5⁺ cells infiltrating the pancreas proliferated less vigorously than 6.5⁺ cells from the lymph nodes of the same mice, probably due to continuous exposure to antigen (Figure 5).

Diabetes onset can be accelerated by cyclophosphamide

Cyclophosphamide has been reported to accelerate diabetes onset in NOD mice (Yasunami and Bach, 1988), but the mechanisms by which it does so remain unclear,

Table I. Percentage of 6.5⁺ cells in the lymph nodes and in the pancreas of TCR-HA×INS-HA mice

	Lymph nodes (% ± SD)	Pancreas (% ± SD)
6.5 ⁺ CD4 ⁺	4.6 ± 1.2	23.0 ± 7.0
6.5 ⁺ CD8 ⁺	5.1 ± 2.0	19.2 ± 5.0

Values represent data obtained by three-color flow cytometry on a total of 16 mice.

Fig. 4. Development of spontaneous diabetes in TCR-HA×INS-HA transgenic mice. Mice were tested weekly for blood glucose, starting at 4 weeks of age and were considered diabetic when their blood glucose levels were ≥ 200 mg/dl for two consecutive measurements. Approximately 30 mice were followed over a period of 4 months. **Fig. 5.** Proliferative response of lymph node cells from a single transgenic TCR-HA mouse, a double transgenic TCR-HA×INS-HA non-diabetic mouse and a double transgenic TCR-HA×INS-HA diabetic mouse. The proliferative response of lymphocytes infiltrating the pancreas of both double transgenic mice is also shown. 2×10^5 cells were cultured with 5×10^5 irradiated BALB/c splenocytes and stimulated with 10 mg/ml of peptide. The number of 6.5⁺ cells per well was determined and proliferation values shown correspond to c.p.m./10³ 6.5⁺ cells. Values for unstimulated cells ranged from 30 to 90 c.p.m./10³ 6.5⁺ cells. The three mice were 8-weeks old and belonged to the same litter. Two other separate experiments gave similar results.

although it has been suggested that it interferes with regulatory pathways (Harada *et al.*, 1984; Yasunami and Bach, 1988). We studied the effect of cyclophosphamide administration in TCR-HA×INS-HA double transgenic mice. One single high dose (200 mg/kg) of cyclophos-

Fig. 6. Cyclophosphamide-induced diabetes in TCR-HA×INS-HA mice on wildtype or on RAG^{-/-} background. Mice were injected i.p. with 200 mg/kg of cyclophosphamide on day zero and monitored for blood or urine glucose levels every third day. Control TCR-HA×INS-HA mice on RAG^{-/-} background were injected with PBS.

phamide induced diabetes in TCR-HA×INS-HA mice, starting at four days after injection. This effect could also be reproduced in double transgenic mice on the RAG^{-/-} background, where all cells express the same TCR (Figure 6). This indicates that class I MHC-restricted T cells are not required for the development of diabetes. In the absence of cyclophosphamide, double transgenic mice on the RAG^{-/-} background develop heavy infiltrates, but seldom progress to diabetes. TCR-HA×INS-HA mice that have been rendered diabetic by cyclophosphamide injection could still live for weeks, similar to observations in mice that become spontaneously diabetic.

Cytokine mRNA expression in total islets of mice with and without diabetes

The expression of the cytokines IFN- γ , TNF- α , IL-4 and IL-10, as well as Fas-ligand (Fas-L), was analyzed by competitive PCR in TCR-HA×INS-HA mice that suffered either from insulinitis only, or from insulinitis as well as diabetes. Transcription levels of each cytokine, expressed as the ratio of the target gene to HPRT gene expression, were determined as described in Materials and methods, and are shown in Table II. The ratio of the corresponding transcription levels from total islets of diabetic versus non-diabetic mice is shown in Figure 7A. The most striking differences were seen with TNF- α and IFN- γ : TNF- α transcription levels were significantly higher in total islets from non-diabetic mice, while IFN- γ was present at higher levels in diabetic mice. Careful examination of the data revealed slight differences with regard to IL-4 and IL-10, however, transcription levels were around the lower detection limit (<0.01). Finally, Fas-L was easily detected in islets from both mice but no significant differences could be observed.

Cytokine expression by antigen-specific T cells

The results obtained by analyzing total islets suggested that there could be differences in the cytokines secreted

Table II. Quantification of cytokine transcripts in total islets cells and 6.5⁺ sorted cells from diabetic and non-diabetic TCR-HA×INS-HA mice

	Total islets		6.5 ⁺ cells	
	Diabetic	Non-diabetic	Diabetic	Non-diabetic
TNF- α	0.10 $\pm$ 0.04	1.05 $\pm$ 0.05	1.85 $\pm$ 0.75	2.15 $\pm$ 0.75
IFN- γ	2.20 $\pm$ 0.20	0.55 $\pm$ 0.05	4.80 $\pm$ 0	0.55 $\pm$ 0.55
IL-4	0.01 $\pm$ 0.01	0.03 $\pm$ 0.03	0.06 $\pm$ 0.03	0.15 $\pm$ 0.05
IL-10	<0.01	0.04 $\pm$ 0.04	0.80 $\pm$ 0	1.50 $\pm$ 0.20

Transcript levels were calculated using competitive RT-PCR as described in the Materials and methods. Values represent the ratio (mean $\pm$ SEM) of target gene to HPRT gene expression of two mice tested per group.

Fig. 7. Comparison of cytokine expression in (A) total islets and (B) 6.5⁺ sorted cells from diabetic versus non-diabetic TCR-HA×INS-HA double transgenic mice. Constant amounts of cDNA samples were amplified in the presence of a multispecific internal plasmid control (pQRS). PCRs were separated on 2% agarose gels, visualized by ethidium bromide staining under UV illumination, and photographed. Image densitometric analysis was performed using NIH Image 1.61 software. Within each panel, the upper band corresponds to the amplification of the competitor, whereas the lower band corresponds to the amplification of experimental cDNAs. Fas-L expression was analyzed by semiquantitative PCR below the saturation stage of amplification. Results are expressed as the mean ratio of cytokine mRNA of two mice with diabetes as compared with two non-diabetic mice. *IL-10 was not detectable (ND) in total islets of diabetic mice and therefore no ratio was calculated.

by the antigen-specific cells that were present in islets of diabetic versus non-diabetic mice. This was confirmed by analyzing 6.5⁺ sorted cells. As shown in Figure 7B and Table II, the transcription levels of mRNA for IFN- γ were ~10-fold higher in the diabetic than in the non-diabetic mice, while the levels of IL-4 and IL-10 were 2- to 3-fold higher in non-diabetic mice. The difference in TNF- α observed in total islets was no longer observed upon analysis of 6.5⁺ cells, suggesting that this cytokine is secreted by non-lymphoid cells present in the islets, such as macrophages. Again, no difference was observed concerning Fas-L expression on antigen-specific cells. These results confirm functional Fas-L-mediated cytotoxicity assays using sorted 6.5⁺ infiltrating cells from the pancreas of diabetic or non-diabetic mice as effector cells, and Fas-transfected L1210 cells as targets: no difference in the lytic capacity of such cells could be found (not shown).

Fas expression by β cells

The possibility that Fas upregulation by β cells could be responsible for the progression from insulinitis to diabetes was examined. For this, immunohistochemistry with the Fas antibody (JO2) was performed on the pancreas of a 5-week-old TCR-HA×INS-HA mouse, which was sacrificed at the beginning of diabetes onset, as well as on the same diabetic and non-diabetic mice, described in Figure 6. As shown in Figure 8, Fas expression could be detected on infiltrating lymphocytes in all types of mice, but expression was not found on the remaining β cells.

Discussion

The TCR-HA×INS-HA double transgenic model is characterized by an early infiltration of the pancreas by lymphocytes, as soon as significant numbers of mature T cells reach peripheral lymphoid tissue. Since the infiltration develops spontaneously, i.e. in the absence of deliberate immunization with exogenous antigens, it appears obvious that the lymphocytes must come into contact with the antigen that is expressed under control of the insulin promoter. This notion is strongly supported by the analysis of lymphocytes in secondary lymphoid organs in double transgenic TCR-HA×INS-HA, versus single transgenic TCR-HA mice. Our analysis of the expression of activation markers after *in vivo* administration with high doses of antigen, clearly indicates that most T cells with the transgenic TCR react differently in double versus single transgenic mice, in that cells from the double transgenic mice express lower levels of Fas, CD25 and CD69, and

Fig. 8. Immunohistochemical analysis of Fas expression in pancreatic islets of TCR-HA $\times$ INS-HA mice. Pancreatic sections were stained with the biotinylated Fas mAb (JO2) and revealed with AEC as described in Materials and methods. Islet β cells, with nuclei (in blue) surrounded by a big cytoplasm, are localized in the center and surrounded by infiltrating lymphocytes with no visible cytoplasm and stained in red. (A) represents a 5-week-old mouse sacrificed at the onset of diabetes, (B) represents an 8-week-old diabetic mouse sacrificed several days after disease onset and (C) represents a non-diabetic mouse of the same litter (magnification 400 $\times$).

react in a way that is less harmful for the animal. This indicates that INS-HA has changed, either directly through contact with antigen and/or indirectly through the release of cytokines, the reactivity of the entire pool of lymphocytes expressing the transgenic TCR. The death caused in single transgenic mice by high doses of antigen may be caused by similar mechanisms to those responsible for lethal shock after injection of superantigens (Miethke *et al.*, 1992), where the activated T cells exhibit similar phenotypic changes, i.e. upregulation of CD25, and where the secretion of TNF- α is involved.

The pancreatic infiltrates in double transgenic mice were large, yet islands of intact insulin-secreting cells could be detected. It is clear that TCR-HA-bearing T cells have an essential function in the formation of islet infiltrates, since there was no infiltration in INS-HA single transgenic mice and since there was a specific enrichment of TCR-HA-bearing cells in the infiltrates. Also, these cells were in a particularly activated state inside, but not outside, the islets, evident by their high expression of CD69 molecules and their low response to restimulation by antigen, which is indicative of recent antigenic encounter. In spite of the huge infiltrates and the abundant presence of activated antigen-specific T cells, the mice did not immediately proceed to becoming diabetic. This may, in part, depend on some regulatory T cells that

prevent the rapid progression, since in our model, as in other diabetes models (Yasunami and Bach, 1988; André *et al.*, 1996), rapid onset of diabetes could be obtained by injection of high doses of cyclophosphamide, even in double transgenic RAG $^{-/-}$ mice, which very rarely become diabetic but nevertheless exhibit huge pancreatic infiltrates. In view of these results, one may argue that if cyclophosphamide interferes with regulatory pathways, then regulatory T cells can express the same receptor as the disease-causing cells. Actually, the mechanism(s) by which cyclophosphamide operates is obscure and perhaps in the future best analyzed in mice that exclusively express the transgenic TCR.

The cellular composition of infiltrates from diabetic versus non-diabetic mice was very similar with regard to number and activation status of infiltrating cells. The analysis of cytokines in the islets of non-diabetic mice indicated that cells in the islets were secreting higher levels of IL-4 and IL-10 than cells in diabetic mice. In contrast, much higher levels of IFN- γ were detected in the islets of mice that did proceed to diabetes. The availability of a clonotypic antibody provided the possibility of determining the origin of these cytokines, by isolating and studying the cytokine profile of the antigen-specific cells, *ex vivo*. The 6.5 $^{+}$ cells of diabetic mice secreted 10-fold higher levels of IFN- γ and somewhat

lower levels of IL-4 and IL-10 than those from mice with insulinitis only. These results suggest that the increase of pro-inflammatory cytokines in the local milieu represents a key factor in modulating the response, such that diabetes develops. A significant difference, which was detected between total islets from diabetic versus non-diabetic mice, concerned TNF- α , which was expressed and produced at higher levels by cells other than the antigen-specific lymphocytes in non-diabetic mice. TNF- α has been found to have a suppressive effect on the incidence of diabetes in adult NOD mice by preventing the development of autoreactive islet-specific cells (Jacob *et al.*, 1990). Also, NOD mice transgenic for TNF- α under the insulin promoter are protected from disease, apparently by mechanisms inducing tolerance toward islet antigens (Grewal *et al.*, 1996). Whether this also applies in our model appears questionable, since the number and proliferative response of the HA-specific T cells appears comparable in the diabetic and the non-diabetic mice. In fact, the transfer of splenic cells, or even infiltrating cells, from non-diabetic mice into immunodeficient INS-HA recipients, can induce diabetes with a similar kinetic to that induced by cells from diabetic mice (A.Sarukhan, unpublished data). It is not clear whether the elevated levels of TNF- α in the non-diabetic mice have any causal relationship to the absence of significant β cell destruction. If so, it would have to act by reversible suppression rather than by inducing irreversible tolerance. Within this context, it should be noted that TNF- α has been reported to lead to the development of ductal endocrine cells capable of secreting enough insulin to prevent development of hyperglycemia, and with altered antigenic specificity in order to escape autoimmune aggression (Higuchi *et al.*, 1992). Furthermore, TNF- α seems to inhibit signaling through the insulin receptor and is associated with the development of a state of insulin resistance, such as that observed in non-insulin-dependent diabetes mellitus (Hotamisligil *et al.*, 1994). Thus, TNF- α could also be operating through these other non-immune mechanisms in the non-diabetic TCR-HA $\times$ INS-HA mice.

IFN- γ and TNF- α both belong to the family of pro-inflammatory cytokines, but in our case only the increased expression of IFN- γ and the decreased expression of TNF- α correlate with the development of diabetes. While, in fact, TNF- α may have some protective effect as discussed above, it could be argued that only the increase in certain pro-inflammatory cytokines has a role in the progression from insulinitis to diabetes. This would be consistent with earlier studies showing that development of diabetes can be inhibited by IFN- γ antibodies in several experimental situations (Campbell *et al.*, 1991; Debray-Sachs *et al.*, 1991) and that IFN- γ deficiency could delay the development of diabetes (Hultgren *et al.*, 1996). Overall, these studies indicate that IFN- γ has an important role in the development of diabetes that may be partially compensated for by other cytokines in IFN- γ deficient mice, a hypothesis that could be tested in conditional knock out mice or in IFN- γ receptor deficient mice. Our study is well in line with these observations (Campbell *et al.*, 1991; Debray-Sachs *et al.*, 1991; Hultgren *et al.*, 1996) and demonstrates directly a correlation between IFN- γ gene expression by antigen-specific cells in the pancreas and the development of diabetes.

While we do see a clear increase in the expression of a pro-inflammatory cytokine gene among antigen-specific T cells, concomitantly with the development of diabetes, we do not know by which effector mechanisms the β cells are destroyed. Recently, Fas was implicated in this pathway in the development of diabetes in NOD mice (Chervonsky *et al.*, 1997). Even more recent studies indicated that Fas was required for early infiltration, since transfer of diabetogenic T cell into Fas-deficient NOD mice did not even lead to infiltration (Itoh *et al.*, 1997). Thus, it is questionable whether Fas on β islet cells has any direct role in β cell death (Benoist and Mathis, 1997). Our data concerning the expression of Fas-L and Fas by infiltrating lymphocytes, as well as β islet cells, indicate that there is no upregulation of Fas on any of the β cells when mice proceed to becoming diabetic. We cannot rule out, however, that diabetes development coincides with low Fas expression not detectable by our technique or is dependent on Fas expression of only those islet cells that are in direct contact with specific T cells. Thus, it appears that further studies are required to determine the molecular mechanisms of β cell destruction.

Materials and methods

Mice

TCR-HA transgenic mice, already described (Kirberg *et al.*, 1994), are on BALB/c background and express a T cell receptor α/β specific for peptide 111–119 from influenza hemagglutinin, presented by I-E^d. INS-HA mice, previously described (Lo *et al.*, 1992), express the hemagglutinin under the rat insulin promoter and are on BALB/c background. TCR-HA mice were backcrossed onto INS-HA mice. The presence of transgenes in offspring was determined by PCR using TCR β primers and HA primers. Mice used were heterozygous for both transgenes. The RAG-^{-/-} double transgenic mice were obtained by crossing RAG-2^{-/-} TCR-HA (H-2^d) mice (described in Kirberg *et al.*, 1994) with RAG-^{-/-} INS-HA mice (H-2^d).

Glucose levels in urine were determined using Glukotest strips (Boehringer Mannheim) and confirmed by blood glucose measurements using BM-Test-Glycemic strips (Boehringer Mannheim). Mice were considered diabetic when blood glucose levels were >200 mg/dl for two consecutive measurements.

Histology

Pancreata were quick frozen in OCT compound, 5 μ m sections were obtained and fixed in acetone for 10 min. Classical hematoxylin/eosin stainings were performed. For immunohistochemistry, biotin-conjugated hamster anti-mouse Fas (clone JO2) was applied on sections for 60–120 min at the appropriate dilution. After washing, slides were incubated with a biotinylated anti-hamster antibody (Vector Laboratories Inc., Burlingame, CA) for 45 min, washed again and finally incubated with streptavidin–peroxidase (Vector Laboratories, Inc.) or with Amdex streptavidin–peroxidase (Amersham, Buckinghamshire, UK). Color reaction was revealed with AEC (Sigma Chemical, St Louis, MO) and the slides were counterstained with hematoxylin. For insulin staining, a guinea pig anti-porcine insulin antibody (Dako, Glostrup, Denmark) and a peroxidase-conjugated anti-guinea pig antibody (Dako) were used as primary and secondary antibodies respectively.

Cyclophosphamide and antigen administration

Cyclophosphamide (Sigma Chemical, St Louis, MO) was diluted in PBS and injected i.p. at a dose of 200 mg/kg. The HA peptide (SVSSFERFEIFPK) was synthesized in the Basel Institute for Immunology. The chimeric immunoglobulin HA-Ig contains the peptide 111–119 of HA in the CDR3 region of the heavy chain, as described (Zaghoulani *et al.*, 1993), and was prepared from the hybridoma supernatant cultured in complete IMDM +3% FCS, previously run over a protein G column (Fast Flow, Pharmacia LKB, Uppsala, Sweden).

Antibodies and FACS analysis

Hybridoma supernatants containing mAbs 6.5 (anti-clonotypic antibody), GK1.5 (anti-CD4), 53-67.2 (anti-CD8), RA3-6B2 (anti-B220) and

M1/70 (anti-Mac-1) were purified by protein G (Fast Flow) affinity chromatography. 6.5 mAb was labeled using fluorescein succinyl ester (FLUOS) (Boehringer Mannheim) according to the manufacturer's instructions. GK1.5 and B220 were biotinylated using Biotin-X-NHS (Calbiochem, La Jolla, CA). The following mAbs were used: FLUOS-labeled 6.5 mAb, anti-CD8-red613 (GIBCO BRL, Gaithersburg, MD), anti-CD4PE (Becton Dickinson, Mountain View, CA), biotin-conjugated anti-CD69 (Pharmingen, San Diego, CA), biotin-conjugated anti-CD25 (Pharmingen), biotin-conjugated anti-CD45RB (Pharmingen), biotin-conjugated anti-CD62L (Pharmingen), SA-PE (Southern Biotechnology Associates Inc, Birmingham, AL) and biotin-conjugated anti-CD95 (Pharmingen). Two- and three-color flow cytometry was performed on a FACScan (Becton Dickinson). Stainings were done in 96 well plates (5×10^5 cells per well) in 10–20 μ l of mAb, at optimal dilution in PBS + 5% FCS + 0.1% azide. Between first and second step reagents, and after the final step, cells were washed in 250 ml of PBS + 5% FCS + 0.1% azide. Data were stored and analyzed with the LYSIS II software (Becton Dickinson).

Isolation of lymphocytes infiltrating the pancreatic islets

Infiltrating lymphocytes were obtained by the technique described by Faveeuw *et al.* (1995). Briefly, individual pancreata were minced with curved scissors in PBS 5% FCS, 1% glucose, 5 mM sodium azide and digested in collagenase P (Boehringer Mannheim), (5 mg/ml PBS + 15% FCS) at 37°C for 2–3 min. The enzymatic reaction was stopped by adding ice-cold medium and three washings. Islets were hand-picked using a dissection microscope and were pressed through a 100 μ m metal sieve. The cell suspension was filtered through a 20 μ m nylon screen. An average of $2\text{--}5 \times 10^6$ cells were obtained per individual pancreas, independently, whether the mice were diabetic or not.

T cell proliferation assays

All *in vitro* assays were performed in complete IMDM [β -mercapto-ethanol (5×10^{-5} M) penicillin (100 IU/ml)] supplemented with 10% FCS. Responder cells were isolated from the pancreas and lymph nodes. Cells (2×10^5 /well) were then cultured with 5×10^5 irradiated (2200 rad) BALB/c splenocytes in the presence of 10 μ g/ml or 1 μ g/ml of peptide or medium alone. ^3H incorporation was measured over the last 18 h of a 66 h culture.

Quantification of cytokine mRNA by competitive RT-PCR

Cytokine mRNA concentrations were determined by reverse transcription followed by competitive PCR, in the presence of defined concentrations of a multispecific internal plasmid control (pQRS). Total islets and 6.5^+ sorted cells from total islets cells were prepared from diabetic and non-diabetic TCR-HA $\times$ INS-HA double transgenic mice as described above. Total RNA was isolated from the cells by using RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription was carried out using Superscript II RT (Gibco-BRL). The primers and PCR conditions for HPRT and cytokine genes (IL-4, IL-10, IFN- γ , TNF- α) and competitive PCR were described previously (Reiner *et al.*, 1994). Fas-L expression was analyzed by semiquantitative PCR, below the saturation stage of amplification. The primers used for Fas-L were: 5'-CGTGAGTT-CACCAACCAAAGC-3' and 5'-CCCAGTTTCGTTGATCACAAG-3'. The cycling conditions for Fas-L cDNA amplification were 94°C for 40 s, 55°C for 1 min and 72°C for 1 min for 30–35 cycles. PCRs were separated on 2% agarose gels, visualized by ethidium bromide staining under UV illumination, and photographed (Polaroid 665 film). Image densitometric analysis was performed using NIH Image 1.61 software (SCR, Bethesda, MD), by integrating the volume in individual amplicons. After subtraction of background values, the density ratio of the competitor band to the target mRNA was determined and relative amounts of cytokine mRNA were calculated based on the starting amount of competitor.

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