A multiplicity of protein antigens in subcellular fractions of rat insulinoma tissue are able to stimulate T cells obtained from non-obese diabetic mice

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Summary. Type 1 (insulin-dependent) diabetes mellitus is a T-cell mediated autoimmune disease with a number of different proteins being implicated as target autoantigens. A 38 kDa protein residing in the insulin secretory granule of insulinoma tissue is recognized by T-cell clones from a newly-diagnosed Type 1 diabetic patient. We have investigated the capacity of normal rat pancreatic beta-cell extracts and various subcellular fractions of transplantable RIN tissue to induce proliferation of T cells from non-obese diabetic (NOD) mice and H-2 identical NON · NOD-H-2^{g7} control mice. Normal rat islet beta-cell protein fractions induced intense, dose-dependent proliferation of NOD splenic T cells, but only marginal proliferative responses of NON · NOD-H-2^{g7} splenic T cells. To further localize the target antigens, four different subcellular fractions from RIN tissue were used as a

source of antigen; here in particular the cytosolic proteins showed dose-dependent activation capacity with splenic T cells in NOD animals. These activities were absent in control mice. There was no proliferation after incubation with microsome preparations from other rat endocrine tissues. Purified carboxypeptidase H did not have any stimulatory activity on NOD T cells. Fractionation of the RIN cytosolic proteins showed a large number of different fractions eliciting proliferative activity. These results demonstrate that NOD T cells respond to a large number of potential islet beta-cell target antigens and it will be necessary to utilize NOD T-cell clones to identify the number and nature of these antigens.

Key words: Type 1 (insulin-dependent) diabetes mellitus, autoimmunity, T cells, rat insulinoma (RIN) tissue.

The inbred non-obese diabetic (NOD) mouse provides a useful animal model for human Type 1 (insulin-dependent) diabetes mellitus [1]. NOD mice spontaneously develop diabetes between 12 and 18 weeks of age with an 80 % predominance in females when bred under germ-free conditions. These animals show clinical and immunological features similar to humans, such as the infiltration of the pancreas by autoreactive mononuclear cells followed by insulitis [1]. There is considerable evidence that the destruction of beta cells is mediated by autoreactive T lymphocytes [2, 3]. T-cell clones that induce diabetes in pre-diabetic animals upon adoptive transfer have been established in a number of laboratories which supports the contribution of autoreactive T cells in the development of the disease [4–8]. However, it is still unclear which proteins are the molecular targets of the infiltrated T lymphocytes leading to beta-cell destruction. A 38 kDa protein in insulin secretory granules of insulinoma tissue has recently been shown to be the target antigen recognized by a human T-cell clone established from a newly-diagnosed Type 1 diabetic patient [9], although the beta-cell specificity of this protein is still not fully proved [9]. In compari-

son to healthy control subjects, a greater number of T-cell lines generated from peripheral blood lymphocytes of Type 1 diabetic patients show reactivity to the 38 kDa secretory granule protein [10]. For further identification of the T-cell targets, we have investigated the reactivity of T cells from spleens and pancreata of diabetic NOD mice to normal rat islet beta-cell proteins and to various subcellular fractions of a transplantable parental rat insulinoma tumour [11]. As a means of establishing the specificity of the T-cell responses obtained to insulinoma subcellular fractions we have used purified splenic T cells from MHC matched, non-diabetic NON · NOD-H-2^{g7} animals.

Materials and methods

NOD animals: Female NOD mice were obtained from Bommice (Bomholtgard Breeding and Research Centre, Ry, Denmark) at the age of 4 or 18 weeks and were maintained in a sterile environment on a conventional diet (incidence of diabetes: 80 % by 16–18 weeks). The non-diabetic NON · NOD-H-2^{g7} female mice were originally bred and maintained at the Diabetes Research Institute, Düsseldorf. In every set of experiments, NOD and NON · NOD-H-2^{g7} mice were age-matched and the NOD mice had the same metabolic status at

Table 1. MHC compatibility of splenic T cells from NON-NOD-H2^{g7} and NOD mice. Irradiated NON-NOD-H2^{g7} spleen cells could replace NOD spleen cells in presenting the antigens of the cytosolic fraction derived from RIN insulinoma tissue

Irradiated APCs	NOD spleen T cells	Cytosolic proteins	cpm $\times 10^{-3}$
NOD	+	+	5.8
NON-NOD-H2 ^{g7}	+	+	7.4
-	+	+	1.2

APCs, Antigen presenting cells

the time of killing, as determined by measurement of portal blood glucose levels using an Eppendorf ACP 50/40 blood glucose meter. Mice with blood glucose levels above 13.9 mmol/l were considered to be diabetic.

Preparation of normal rat pancreatic islets: Normal rat pancreatic islets were purified by hand picking after collagenase digestion [12]. Approximately 2500 highly purified islets were disrupted by freeze thaw lysis as follows: pelleted frozen islets were resuspended in 0.5 ml sterile phosphate-buffered saline (136.8 mmol/l NaCl) containing a cocktail of protease inhibitors (100 µg/ml phenylmethylsulphonyl fluoride, 100 µg/ml aprotinin, 10 µg/ml leupeptin and 10 µg/ml pepstatin) and allowed to thaw slowly on ice. After gentle mixing with a 1 ml Gilson pipette, the sample was subjected to a second cycle of freezing and thawing. After a brief centrifugation step (2000 g for 10 min at 4°C) to remove nuclei and unbroken cells, the supernatant was ultracentrifuged at 45,000 g for 1 h at 4°C. The supernatant was termed the cytosolic fraction. The pelleted microsomal membranes were washed two times by resuspending in a glass homogenizer in ice cold phosphate buffered saline and ultracentrifugation as described. Protein was measured by the Pierce protein assay.

Preparation and fractionation of transplantable rat insulinoma tissue (RIN): Subcellular fractions from RIN tissue were prepared as described [13]. Briefly, transplantable RIN tissue was homogenized in isotonic sucrose buffer, centrifuged to remove cell debris and nuclei and fractionated by density gradient centrifugation on Nycodenz gradients into fractions enriched in light membrane (composed chiefly of plasma membrane), secretory granule components (crude granules), pellet (composed of endoplasmic reticulum, lysosomes and mitochondria) and a fraction comprising cytosolic proteins. The nature of each of the latter three membrane fractions was verified by enzymatic analysis of marker proteins localized within each of the intracellular compartments, as described in detail previously [9]. The RIN cytosolic and the microsomal membrane pellets were fractionated by gel electrophoresis and electroelution into 18 different molecular weight fractions ranging from over 120 kDa to 14 kDa as already described [9]. Each different subfraction (at 2 to 10 µg/ml) was tested for its ability to induce proliferative activity of NOD T cells in a dose-responsive manner at three different dilutions (1:50, 1:200 and 1:500).

Isolation and culture of splenic T lymphocytes: For each experiment 4–5 diabetic female NOD mice or 4–5 female NON-NOD-H2^{g7} mice of the same age, or both, were killed. The animals were non-fasting. Spleens of NOD and of NON-NOD-H2^{g7} mice were removed aseptically and a homogenous cell suspension prepared. All cell work was performed in complete RPMI-1640 medium (RPMI-1640, supplemented with 10% fetal calf serum, 10 units/ml penicillin/streptomycin, 200 mmol/l L-glutamine and 5×10^{-5} mol/l β -mercaptoethanol). Pooled cell suspensions of spleens were separately layered on Ficoll-400 cushions (Lymphoprep; Boehringer Mannheim, Mannheim, FRG) and centrifuged for 45 min at 10,000 g. The interface containing mononuclear cells was recovered, washed and passed over nylon wool columns to obtain purified T cells. Triplicate assays were carried out by culturing 10^4 purified T cells in a total volume of 200 µl of complete RPMI-1640 in 96-well microtitre plates in the presence of 10^5 irradiated (3000 rad) syngeneic spleen cells as antigen presenting cells (APC). For proliferation

assays, all protein fractions were used at final dilutions of 1:20, 1:50, 1:250 and 1:1000 (representing approximate final concentrations in the range of 50 µg/ml to 1 µg/ml) to determine their respective optimal concentration for stimulation of the T cells. Highly purified carboxypeptidase H isolated from the RIN tissue [14] was used at 100, 10, 1 and 0.1 µg/ml. As a control for T-cell blastogenesis activity, in each case concanavalin A (1 µg/ml) was used whilst the basal stimulatory activity was ascertained in the presence of complete cell culture medium without antigen. The cultures were incubated for 72–86 h, then pulsed with 1 µCi per well ³H-methyl-thymidine (³H-TdR) (Amersham Buchler, Braunschweig, FRG) and harvested 20 h later. The incorporated radioactivity was determined using a Betaplate scintillation counter. Proliferation was expressed as stimulation index (SI): lymphocyte DNA synthesis/³H-TdR incorporation/counts per min (cpm) in the presence of antigen over cpm in the absence of antigen.

Immunodepletion of cytosolic fraction of insulin and GAD₆₅: Radioimmunoassay analysis for the presence of insulin and C-peptide in the cytosolic fraction was performed using kits from CIS-Bioindustries, Gif-Sur-Yvette, France; and Boehringer, respectively. For immunodepletion, monoclonal antibodies to insulin (CIS-Bioindustries) and to GAD₆₅ [15] were individually bound to protein G sepharose followed by covalent linking. The cytosolic fraction was incubated separately with the antibody affinity columns for 24 h on a roller shaker at 4°C. The efficiency of the immunodepletion was determined by dot blotting. Before and after immunodepletion 7 µl of the cytosolic fraction was blotted on a nitrocellulose membrane and incubated with anti-insulin or anti-GAD₆₅ antibodies. After incubation with the appropriate second antibody conjugated to horseradish peroxidase, antibody binding was determined by chemiluminescence assay (ECL, Amersham). The insulin and GAD₆₅ immunodepleted cytosolic fractions were used for proliferation experiments as already described.

Statistical analysis

Data are shown as means + standard deviation (SD) from at least three identically performed experiments. One-way analysis of variance and the Kruskal-Wallis test were used to assess the results. Differences with *p* values of < 0.001 were considered to be significant.

Results

The compatibility of the NOD and the NON-NOD-H2^{g7} control mice used in our studies was investigated by mixed lymphocyte reaction (MLR) experiments. Irradiated spleen cell APC from NOD or NON-NOD-H2^{g7} did not lead to any appreciable stimulation of NOD splenic cells in MLR assays (0.8×10^{-3} cpm with APC derived from NOD and 0.5×10^{-3} cpm with APC derived from NON-NOD-H2^{g7} mice) indicating that there were no subtle differences in the MHC haplotypes of these animal strains (data not shown). Furthermore, replacement of irradiated NOD spleen APC by spleen cells from NON-NOD-H2^{g7} for presentation of the cytosolic proteins to NOD T cells slightly increased the NOD T cell proliferative responses (Table 1).

Splenic T-cell reactivity of NOD and NON-NOD-H2^{g7} to pancreatic islet beta cells was examined using fractionated rat islet proteins. The pancreatic islets purified by hand picking after collagenase digestion [12] were disrupted by freeze thaw lysis and the cytoplasmic extract and a crude microsomal membrane fraction were obtained. Splenic T cells from diabetic NOD mice (23 weeks old)

Fig. 1. a) T-cell responses of NOD (■) and NON·NOD-H-2^{g7} (▨) mice to normal rat islet protein and microsomal proteins from other rat tissues. Proliferation of T-cells, expressed as stimulation index, from 20–23-week-old diabetic NOD mice was significantly increased ($p < 0.001$) after stimulation with normal pancreatic islet beta-cell cytoplasm (BCC) and islet beta-cell microsomal membrane (BCMM). Stimulation with microsomal membrane fractions from other rat tissues such as whole pancreas homogenate (WP), thyroid microsomes (T), adrenal gland microsomes (AG), pituitary gland microsomes (P) did not lead to any significant T-cell proliferative activity. **b)** T-cell dose-dependent response of NOD and NON·NOD-H-2^{g7} mice to normal rat islet cell protein and various subcellular fractions of the RIN tissue. The columns show the stimulation index of the proliferation of splenic T cells isolated from NOD (■) or NON·NOD-H-2^{g7} mice (▨) to various concentrations of these antigens

showed strong proliferative responses to both the beta-cell cytosolic (stimulation index (SI) = 59) and microsomal membrane fractions (SI = 47.3), while the non-diabetogenic NON·NOD-H-2^{g7} mice (20 weeks old) showed considerably reduced T-cell responses to these normal rat islet beta-cell proteins (SI = 10.6 and 6.7 to the cytosolic and the membrane fraction, respectively) (Fig. 1a). T cells from neither NOD nor NON·NOD-H-2^{g7} mice showed any proliferative responses to rat whole pancreatic extract, rat thyroid microsomes, rat adrenal gland microsomes or rat pituitary microsomes (all SI-values < 1.8) (Fig. 1a). As shown in Figure 1b, maximum optimal proliferative responses for NOD spleen T cells were apparent at 3.4 μg/ml and 24 μg/ml for beta-cell cytoplasmic and microsomal membrane proteins, respectively, whilst for splenic T cells derived from NON·NOD-H-2^{g7} mice maximal responses were obtained at 34 μg/ml and 1.8 μg/ml, respectively. The extent to which each of the RIN subcellular fractions induced T-cell proliferative activity are shown in Table 2. The cytosolic protein fraction elicited the highest proliferation in spleen T cells purified from diabetic NOD mice; some proliferative reactivity was present to the pellet and the crude secretory granule fractions, whilst there was no reactivity to the insulinoma plasma membrane (light membrane). Maximum optimal proliferative responses were apparent at 7.6 μg/ml, 0.99 μg/ml and 25 μg/ml for the RIN

cytoplasmic, secretory granule and pellet fractions, respectively (Fig. 1b). When spleen T-cell responses from NON·NOD-H-2^{g7} mice to the four subcellular fractions were analysed, marginal responses were obtained to the RIN cytosolic proteins, pellet, the crude granules and the light membrane fraction (SI = 4 ± 1.9 , 2.6 ± 0.8 , 3.8 ± 1.9 , 3.1 ± 2.2 , respectively) (Table 2 and Fig. 1b).

Using T cells isolated from spleens of young, non-diabetic NOD mice (6 to 8 weeks old), proliferation was

Table 2. Proliferation of T cells from NOD and control mice after stimulation with four different subcellular fractions from the RIN transplantable insulinoma tissue. Stimulation with Concanavalin A (Con A) was used as a positive control

Stimulant	Purified T cells from	
	NOD spleen	NON·NOD-H2 ^{g7} spleen
Cytosolic proteins	38.8 ± 20.6	4.0 ± 1.9
Microsomal membrane/pellet	16.5 ± 7.5	2.6 ± 0.8
Crude granules	10.6 ± 3.3	3.8 ± 1.9
Light membranes	2.5 ± 0.9	3.1 ± 2.2
Con A	160.0 ± 43.4	140.3 ± 13.4

Proliferation is expressed in stimulation index (SI) values ± SD

still present with SI-values of 23.1 ± 5.1 and 13.5 ± 5.7 for cytosolic and pellet fractions, respectively. Proliferative responses to T cells from age- and sex-matched non-diabetic control mice to these two fractions was significantly reduced (Fig. 2).

Further experiments on the characterization of the T-cell stimulating proteins in these fractions was performed on the material derived from RIN tissue due to the availability of larger quantities of protein samples available. We established whether pro-insulin and glutamate decarboxylase (GAD) were also present in the subcellular fractions, and whether they played a role in the NOD T-cell responses described above. Radioimmunoassay analysis of the cytosolic fraction showed the presence of insulin (2.46 pmol/ml), whilst C-peptide was not detectable. SDS-polyacrylamide gel analysis of the cytosolic protein fraction showed a large number of proteins ranging from over 200 to 10 kDa (Fig. 3a). Using monoclonal antibodies to

Fig. 2. Increased proliferation of splenic T cells from 6-8-week-old, non-diabetic NOD (■) mice compared to T cells from age-matched NON-NOD-H2^{b7} (▨) control mice after incubation with RIN cytosolic and pellet fractions

GAD₆₅ [15], GAD₆₅ was detectable in the RIN cytosolic fraction by dot blot analysis (Fig. 3b). By serial immunodepletion experiments the rat insulinoma cytosolic preparation was immunodepleted of insulin (to 0.05 pmol/ml) and GAD₆₅ polypeptides (Fig. 3b). The insulinoma cytosolic proteins depleted of insulin and GAD₆₅ maintained their ability to induce proliferation of the NOD splenic T-cell population (Fig. 3c). Highly purified carboxypeptidase H failed to induce any T-cell stimulation in NOD mice (Fig. 3c).

For further definition of the relevant antigens in the RIN fractions, the protein components of the RIN cytosolic fraction (Fig. 3a) and of the pellet were separated by preparative gel electrophoresis and electroelution techniques. The obtained fractions were assessed for NOD T-cell stimulatory activity by determining their respective optimal concentration in dose response assays. Using 18 different electroeluted fractions comprising of different molecular weights encompassing over 120 kDa to 14 kDa [9], we found a complex pattern of reactivity involving many antigens derived from the cytosolic and membrane pellet fractions that elicited proliferative activity in NOD splenic T cells (Fig. 4).

Discussion

It has been suggested, that proteins of the insulin secretory granules are transiently exposed on the cell surface and can therefore interact as antigens with the immune system in Type 1 diabetes [9]. Our study was performed to determine if there are proteins other than the known antigens such as GAD, insulin, proinsulin and the 38 kDa within the cytosolic compartments and the insulin secretory granules present eliciting spontaneous T-cell responses in NOD mice.

The cytosolic fractions and the crude microsomal membrane pellets derived from both the rat islets and the transplantable RIN insulinoma induced T-cell prolifer-

Fig. 3(a-c). Immunodepletion of the insulinoma cytosolic fraction of insulin and glutamate decarboxylase (GAD) and its affect on the NOD T-cell proliferative activity **a)** SDS polyacrylamide gel electrophoresis (10% gel) of the insulinoma cytosolic fraction stained for protein with Coomassie brilliant blue. The molecular weights are indicated. **b)** Dot blot hybridization of the cytosolic fraction before and after serial immunodepletion of insulin and GAD protein. **c)** NOD splenic T-cell proliferation with the insulinoma cytosolic preparations before (■) and after (□) immunodepletion of insulin and GAD proteins by immunoaffinity chromatography. The effect of purified carboxypeptidase H (▨) from RIN cells is also shown

Fig. 4. Proliferation of splenic NOD T cells with fractionated proteins derived from RIN insulinoma cytoplasmic (---x---) and microsomal membrane (—) fractions after stimulation with 18 different subfractions. The fractions were electroeluted from SDS polyacryl-

amide gel strips of different molecular weights in the range from 12.5–120 kDa. Three different concentrations of each of these fractions were tested and the highest stimulation index (SI) values obtained at one of these concentrations was plotted

erative activity in NOD mice, with a similar dose response of between 3.5–7.5 and 25–42 µg/ml, respectively. However, the rat islet beta-cell proteins were also capable of inducing proliferative responses from T cells isolated from the non-diabetogenic NON·NOD-H-2^{g7} mice, although these responses were significantly lower than those observed in the NOD animals. The fact that these control mice did not show T-cell responses to any of the insulinoma subcellular fraction suggests that the responses of the NOD T cells are related to disease activity and also eliminates the possibility that the NOD T-cell reactivity is due to a tumour-specific antigen.

The NOD spleen T-cell responses to the various insulinoma subcellular fractions were obtained from animals of 18 to 24 weeks of age. However, islet-infiltrating T cells can be detected at the age of 6 to 8 weeks [16–18]. When T cells from non-diabetic 6–8-week-old NOD mice, or 13-week-old NOD mice (data not shown), were used, proliferation was still present, indicating that the islet autoantigen specific T-cell autoreactivity is a long-standing phenomenon in the NOD animals, present long before the precipitation of diabetes.

It is possible that the proteins in the pellet and the crude granule fractions which also give weak NOD T-cell responses, could be due to contaminating proteins from

the cytosol. If this is indeed the case, then these contaminating proteins must be very tightly associated, since extensive washing in high salt buffers of the pellet and crude granule membranes did not abolish their ability to induce NOD T-cell proliferative responses (not shown).

In Type 1 diabetes in man, a number of potential target autoantigens such as insulin and proinsulin [1, 19], GAD [20], carboxypeptidase H [21], heat shock proteins (HSP) [22] and the aforementioned 38 kDa antigen [9] have been proposed to be the relevant pathogenetic autoantigens and indicate the current uncertainty in this field. The fact that subcellular fractions from other neuroendocrine rat tissues which also contain the 38 kDa antigen [9] did not lead to any appreciable NOD T-cell stimulation is indicative that at least in the NOD animals the 38 kD antigen may not be the target T-cell antigen. Carboxypeptidase H has been proposed as a potential target autoantigen in human Type 1 diabetes based on the presence of autoantibodies in these patients. However, as shown here, carboxypeptidase H does not appear to be a target for T cells in NOD animals. Also it has been stated previously that insulin is unable to induce T-cell responses in diabetic NOD mice [19]. In our study the cytosolic fraction immunodepleted from insulin still elicited NOD T-cell reactivity. Our serial immunodepletion experiments with proinsulin and GAD₆₅ indi-

cate that other proteins are present in the cytosolic preparations which are recognized as antigens by the NOD T cells. However, it is still possible that the stimulatory effect of the beta-cell fractions at least partly derives from GAD₆₅ or the 38 kDa protein as purified preparations of these two proteins were not examined in this study. It further cannot be excluded that smaller peptides derived from GAD₆₅ which are unable to bind to the affinity column may still be present in the immunoprecipitated fractions and are recognized by the T cells. This might also be the case using electroeluted protein samples. Proteolytic degradation products in the lower molecular weight fraction derived from a very small number of the relevant antigens possibly elicit NOD T-cell responses.

In summary, our results demonstrate that T cells from NOD mice respond intensively to cytosolic and microsomal membrane fractions from normal rat islets and from the RIN cells. Insulin, proinsulin or carboxypeptidase H do not appear to play a role in the induction of T-cell proliferation in NOD mice. It cannot be fully excluded that GAD₆₅ acts as an antigen, but according to our results there might be others. Experiments using electroeluted proteins suggest that there may be a multiplicity of antigens involved in T-cell stimulation. Recently, islet cell antibodies identified in Type 1 diabetic patients have been shown to give a heterogeneous pattern of staining by indirect immunofluorescence on sections of human pancreas, suggesting that multiple islet cell antigens are also recognized by these autoantibodies [23]. Multiple antigens on beta cells recognized by T-cell populations have been implicated in the pathogenesis of diabetes in NOD mice [6, 24]. Clearly in further defining the nature of these antigens, T-cell clones generated from diabetic NOD mice will be the necessary approach to characterize the T-cell related beta-cell antigens.

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