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Immunology

Peripherin: An islet antigen that is cross-reactive with nonobese diabetic mouse class II gene products

(autoimmunity/diabetes/peripherin)

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ABSTRACT **The nonobese diabetic (NOD) mouse, in which major histocompatibility complex genes may be involved in the susceptibility to diabetes, has been developed as a model of autoimmune diabetes. The NOD mouse expresses I-A-encoded class II major histocompatibility complex antigens, which differ from those of other mouse haplotypes by the presence of a serine at position 57 of the A_β chain. Identifying islet autoantigens may help elucidate the role of class II antigens in the activation of autoreactive T cells and, thus, in the development of diabetes. We have detected autoantibodies directed against a 58-kDa islet cell antigen in NOD mice but not in other strains, including lupus-prone mice. Apart from insulin-secreting cells, the 58-kDa antigen was only found to be expressed by neuroblastoma cells and was identified as peripherin, an intermediate filament protein previously characterized in well-defined neuronal populations. This autoantigen cross-reacted with I-A^{nod} class II antigens, suggesting that it may contribute to defective self-tolerance of islet β cells in the NOD mouse.**

The nonobese diabetic (NOD) mouse develops early insulitis and spontaneous insulin-dependent diabetes mellitus, mostly affecting females >10 weeks of age (1). The role of T cells in this model is shown by the predominance of T lymphocytes within the islet infiltrate (2, 3), the preventive effect of neonatal thymectomy (4) and treatment with anti-CD4 (5) or anti-class II (6) monoclonal antibodies, and the induction of diabetes by transfer of T cells from diabetic NOD mice to nondiabetic B-cell-deprived NOD recipients (7). Genetic studies have pointed to the role of major histocompatibility complex (MHC) genes in the susceptibility to diabetes (8, 9). The NOD mouse expresses only I-A-encoded class II antigens, which differ from those of other mouse haplotypes by the presence of a serine at position 57 of the external domain of the A_β chain (10, 11). MHC-associated susceptibility or resistance to diabetes may be related to the role of class II antigens in the presentation of autoantigenic peptides, selection of the developing T-cell repertoire, or the control of peripheral regulatory T cells (12). A key issue in understanding the link between class II MHC antigens and the activation of autoreactive T cells is the identification of β-cell target autoantigens. Circulating autoantibodies provide a probe for identifying target autoantigens and have been detected in the NOD mouse by means of immunoprecipitation, Western blot analysis, and indirect immunofluorescence (13–15). In diabetic patients, autoantibodies against a 64-kDa islet protein (16) and glycolipids (17) have been identified. Although the role of autoantibodies in β-cell destruction is minor relative to that of T cells (7), autoantibodies may help to identify autoantigens to which self-tolerance is defective.

We report the detection in NOD mice of circulating autoantibodies directed against a 58-kDa islet antigen identified as peripherin, an intermediate filament protein previously characterized in well-defined neuronal cells. Since peripherin is cross-reactive with class II MHC molecules expressed at the I-A locus in the NOD mouse, it may contribute to defective self-tolerance and the immune destruction of insulin-secreting cells in this model.

MATERIALS AND METHODS

Mice. NOD mice were bred in our facilities under specific pathogen-free conditions and checked every 6 months for bacterial, viral, and parasitic infections. The spontaneous incidence of diabetes in our colony is about 55% in females and <5% in males at 6 months of age. Mice were monitored for glycosuria three times a week (Glukotest; Boehringer Mannheim) and for fasting glycemia using test strips and a colorimetric assay (Haemoglucotest and Reflolux F; Boehringer Mannheim) when glycosuric. Diabetes was diagnosed when permanent fasting glycemia > 3 g/liter occurred. Swiss nude mice were used to produce hybridoma ascites (Iffa Credo, St. Germain sur l'Arbresles, France).

Cell Preparations. Rat insulinoma Rin5F cells (18), rat thymic epithelial cells (19), and L929 rat fibroblast cells (20) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin at 100 units/ml, and streptomycin at 100 μg/ml (GIBCO/BRL). Normal islets were prepared by collagenase (Sigma) digestion of mouse pancreases as described by Lacy and Kotianovsky (21) with slight modifications (22).

Western Blots. Cells (1×10^8) from each cell line were washed in Hanks' balanced saline solution, suspended in 7 ml of 0.01M Tris-buffered saline (TBS)/10 mM $CaCl_2$/0.25 M sucrose, pH 7.4, and disrupted at 4°C. The cell homogenates were centrifuged (1000 × *g*, 10 min), and the supernatant was subjected to ultracentrifugation (20,000 × *g*, 1 h). The pellet was then resuspended in 2.3% SDS/62.5 mM Tris/10% glycerol/5% 2-mercaptoethanol, pH 6.8. One-dimensional gel electrophoresis and electrotransfers to nitrocellulose filters were performed as described by Laemmli (22) and Burnette (23). Two-dimensional SDS/PAGE was performed as described by O'Farrell (24). The filters were soaked in 0.02 M TBS/3% gelatin, washed in 0.05% Tween 20/0.02 M TBS, and then incubated with the test sera or ascitic fluid diluted 1:50 in 1% gelatin/0.05% Tween 20/0.02 M TBS. After washing, they were incubated with biotinylated sheep anti-

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Abbreviations: NOD, nonobese diabetic; TBS, Tris-buffered saline; PBS, phosphate-buffered saline; NCS, newborn calf serum; MHC, major histocompatibility complex.

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mouse immunoglobulin (diluted 1:100; Amersham), followed by streptavidin–biotinylated-peroxidase complex (1:150; Amersham), and then stained with 4-chloro-1-naphthol. The molecular weight markers used were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400) (Pharmacia).

RIA. Monoclonal antibodies in ascitic fluid were used at dilutions of 1:100 or 1:1 × 10^8 in 100 μl of phosphate-buffered saline (PBS) supplemented with 5% newborn calf serum (NCS) and incubated with 10^5 Rin5F or spleen cells or with 2 × 10^4 DBA/2 islet cells for 1 h at room temperature in 96-well plates precoated with 20% NCS in PBS. After four washings, the cell pellets were incubated with goat anti-mouse immunoglobulins in 100 μl of 5% NCS in PBS and then with ^{125}iodine-labeled protein A in 100 μl of 5% NCS in PBS. Radioactivity contained in the pellet was determined after further washings.

Monoclonal Antibodies. Anti-class II monoclonal antibodies were kindly provided by H. O. McDevitt (Stanford, CA) and have been characterized as anti-I-$E^{k,d,p,r}$ (14.4.4), anti-I-A^d (MKD6), anti-I-$A^{b,d,q,u,j}$ (BP107), anti-I-A^k ($A\alpha^k$, 116-32), and anti-I-$A^{f,k,r,u,s,nod}$ ($A\beta^k$, 10-2-16 and 10-3-6) (25–28). Anti-islet monoclonal autoantibodies were obtained by fusing diabetic NOD spleen cells with NS-1 cells according to Köhler and Milstein (29).

Absorption Experiments. Peripherin was purified from Rin5F cells suspended in PBS containing 0.5 mM phenylmethylsulfonyl fluoride by disruption in 10 mM Tris/10 mM NaCl/1% Triton X-100/5 mM EDTA, pH 7.4. After centrifugation (10,000 × *g*, 10 min), the pellet was homogenized in 10 mM Tris/10 mM NaCl/1.5 M KCl/0.5% Triton X-100/5 mM EDTA, pH 7.4, and incubated at 4°C for 90 min. After centrifugation, the pellet was suspended in 10 mM Tris/1 mM EDTA/8 M urea/0.5 mM phenylmethylsulfonyl fluoride. Purity was assessed by one-dimensional gel electrophoresis. Absorption of anti-peripherin autoantibody was performed by incubating 100 μl of a 1:50 dilution of 72-2 ascitic fluid (4 μg) with 250 μl of purified peripherin (10 μg) in the above buffer for 18 h at 4°C.

RESULTS

Immunoblot Detection of anti-58 kDa Antibodies in NOD Mouse Sera. Using Western blot analysis of Rin5F rat insulinoma cell extracts, we detected circulating antibodies against a 58-kDa antigen in sera from all the diabetic and nondiabetic 6-month-old NOD mice tested. By contrast, no anti-58-kDa antibodies were detected in sera from common laboratory strains, including autoimmune (NZB × NZW)F_1 mice (Fig. 1). Anti-58-kDa antibodies were also detected in 8-week-old but not in 3-week-old NOD mice (Table 1).

Hybridomas were obtained by fusing NS-1 mouse myeloma cells with spleen cells from a female NOD mouse with recent-onset diabetes. Hybridoma supernatants were screened by Western blot analysis of Rin5F cell extracts. The monoclonal antibody 72-2 was selected as reacting with a 58-kDa antigen and subcloned. The absence of reactivity of 72-2 NOD monoclonal autoantibody with murine thymic epithelial cells and fibroblast extracts (Fig. 1) as well as GH3 pituitary and P815 mastocytoma cells (data not shown) indicated that the 58-kDa antigen was preferentially expressed by islet-related cells.

Reactivity of the 58-kDa Antigen with Anti-Class II Antibodies. We next evaluated the reactivity of the 58-kDa antibody with unrelated monoclonal antibodies, including anti-class II antibodies. Two anti-class II antibodies (10-2-16 and 10-3-6), characterized by their specificity for H-2^k and NOD class II-expressing spleen cells, were shown to cross-react with Rin5F rat insulinoma cells by means of an RIA.

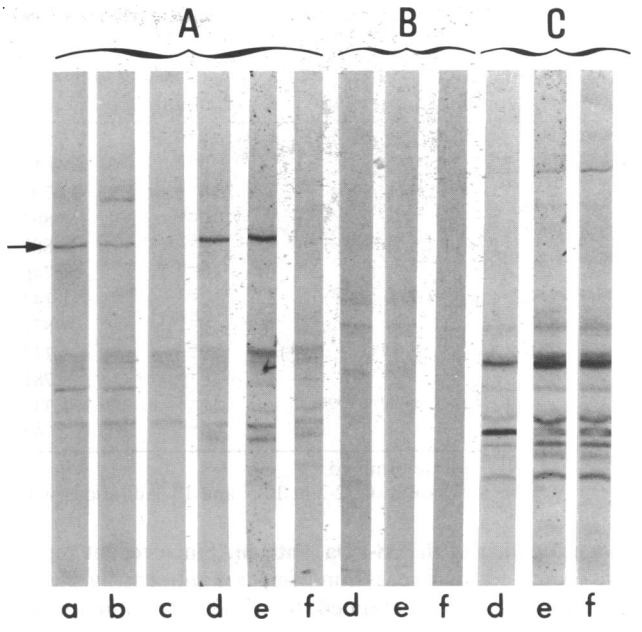

FIG. 1. Western blot analysis of NOD mouse sera and monoclonal antibodies. Cell extracts were prepared from Rin5F cells (*A*), rat thymic epithelial cells (*B*), and L929 fibroblasts (*C*), as well as from GH3 pituitary cells, P815 mouse mastocytoma cells, and human Raji cells (not shown), and blotted on nitrocellulose filters. Cell extracts in *A*, *B*, and *C* were then tested for reactivity with diabetic NOD mouse serum (lane a), nondiabetic 6-month-old NOD mouse serum (lane b), 8-week-old CBA mouse serum (lane c), or monoclonal antibodies 72-2 (lanes d), 10-2-16 (lanes e), or MKD6 (lanes f). Other strains tested (not shown) included BALB/c, C3H, CBA, and (NZB × NZW)F_1 mice. The arrow indicates the 58-kDa bands.

Four other mouse anti-class II monoclonal antibodies with well-defined specificities did not react with NOD spleen cells and did not bind to Rin5F rat insulinoma cells. Importantly, the 58-kDa antigen recognized by anti-I-A^{nod} monoclonal antibodies was expressed by normal DBA/2 mouse islet cells. Direct evidence that the autoantigen recognized by anti-I-A^{nod} antibodies on Rin5F and islet cells differed structurally from NOD class II antigens was obtained in the same RIA, showing that 10-3-6 (Ia17, anti-H-$2^{k,r,u,s,f,nod}$) did not react with DBA/2 (H-2^d) spleen cells but did react with normal DBA/2 islet cells (Table 2). Importantly, the 72-2 monoclonal antibody did not react with NOD spleen cells, which express I-A antigen in the RIA (footnote to Table 2) or in testing immunoprecipitation products of [^{35}S]methionine-labeled NOD spleen cells in two-dimensional SDS/PAGE (data not shown). As shown by Western blot analysis of Rin5F insulinoma cell extracts, the 10-2-16 anti-I-A^{nod} antibody reacted with a 58-kDa antigen at the same position as that described above for the 72-2 NOD monoclonal autoantibody (Fig. 1). Furthermore, NOD mouse sera 72-2 and 10-2-16 recognized the same 58-kDa acidic spot on Western blots of Rin5F cell extracts separated by two-dimensional SDS/PAGE (Fig. 2).

Table 1. Detection of anti-58-kDa autoantibodies in sera from NOD mice of different ages or metabolic status

Parameter	Normoglycemic NOD mice			Diabetic NOD mice
	3 weeks	8 weeks	24 weeks	
No. of animals	5	21	12	15
Anti-58 kDa positive* mice	0	18	12	15

*Anti-58-kDa antibodies were detected in the serum by Western blot analysis of Rin5F cell extracts as described in *Materials and Methods* and Fig. 1.

Table 2. Reactivity of anti-class II monoclonal antibodies with spleen and islet cells

Antibody (specificity)	Ascites dilution	DBA/2 spleen cells (H-2^d), cpm	CBA spleen cells (H-2^k), cpm	NOD spleen cells* (H-2^{nod}), cpm	Rin5F cells, cpm	DBA/2 islet cells, cpm
14-4-4 (I-$E^{k,d,p,r}$)	10^{-2}	6,566	9,702	1,316	227	644
	10^{-8}	658	1,311	1,709	116	ND
MKD6 (I-A^d)	10^{-2}	13,795	1,213	1,390	247	597
	10^{-8}	862	1,314	1,333	249	ND
BP107 (I-$A^{b,d,q,u,j}$)	10^{-2}	8,882	1,389	2,520	115	ND
	10^{-8}	746	1,116	738	152	ND
116-32 (I-A^k, $A\alpha^k$)	10^{-2}	1,143	6,132	1,040	308	ND
	10^{-8}	871	1,114	1,049	141	ND
10-2-16 (I-$A^{f,k,r,u,s,nod}$, $A\beta^k$)	10^{-2}	1,112	8,657	12,835	2401	951
	10^{-8}	781	1,023	829	99	ND
10-3-6 (I-$A^{f,k,r,u,s,nod}$)	10^{-2}	1,331	14,244	18,917	2325	3855
	10^{-8}	764	1,325	875	102	ND

ND, not determined.
*The incubation of 72-2 at 10^{-2} and 10^{-8} dilutions with NOD spleen cells gave respective counts of 1445 and 833.

Identification of the 58-kDa Antigen. Since recent reports have indicated the expression of neural antigens by pancreatic islet cells, we screened the 72-2 NOD monoclonal autoantibody with a wide variety of tissue preparations, including neuronal cells. Two-dimensional SDS/PAGE of neuronal cell extracts identified a previously characterized intermediate filament protein reacting with NOD sera, 72-2 monoclonal autoantibody, and anti-I-A^{nod} antibodies. The antigen recognized by NOD sera, 72-2, and anti-I-A^{nod} antibodies was in precisely the same position as that identified as peripherin on total cell extracts (Fig. 2). Preincubation of 72-2 monoclonal autoantibody with purified peripherin gave complete absorption of anti-58-kDa monoclonal antibody as evidenced by testing of a preabsorbed preparation on Western blots of Rin5F cell extract (Fig. 3).

Cross-Blocking Experiments. Cross-blocking experiments were performed on Rin5F Western blots to further characterize the cross-reactivity shown by NOD autoantibodies, 72-2 NOD monoclonal antibody, and anti-I-A^{nod} monoclonal antibodies. The reaction of biotinylated 72-2 NOD autoantibody with the 58-kDa antigen was not modified by an excess of unlabeled 10-2-16, suggesting that the NOD autoantibody and anti-I-A^{nod} antibodies recognize different epitopes on the 58-kDa islet antigen (Fig. 4).

DISCUSSION

We report the detection of autoantibodies directed against a 58-kDa antigen expressed by Rin5F rat insulinoma cells and normal mouse islets in sera from diabetic NOD mice. Circu-

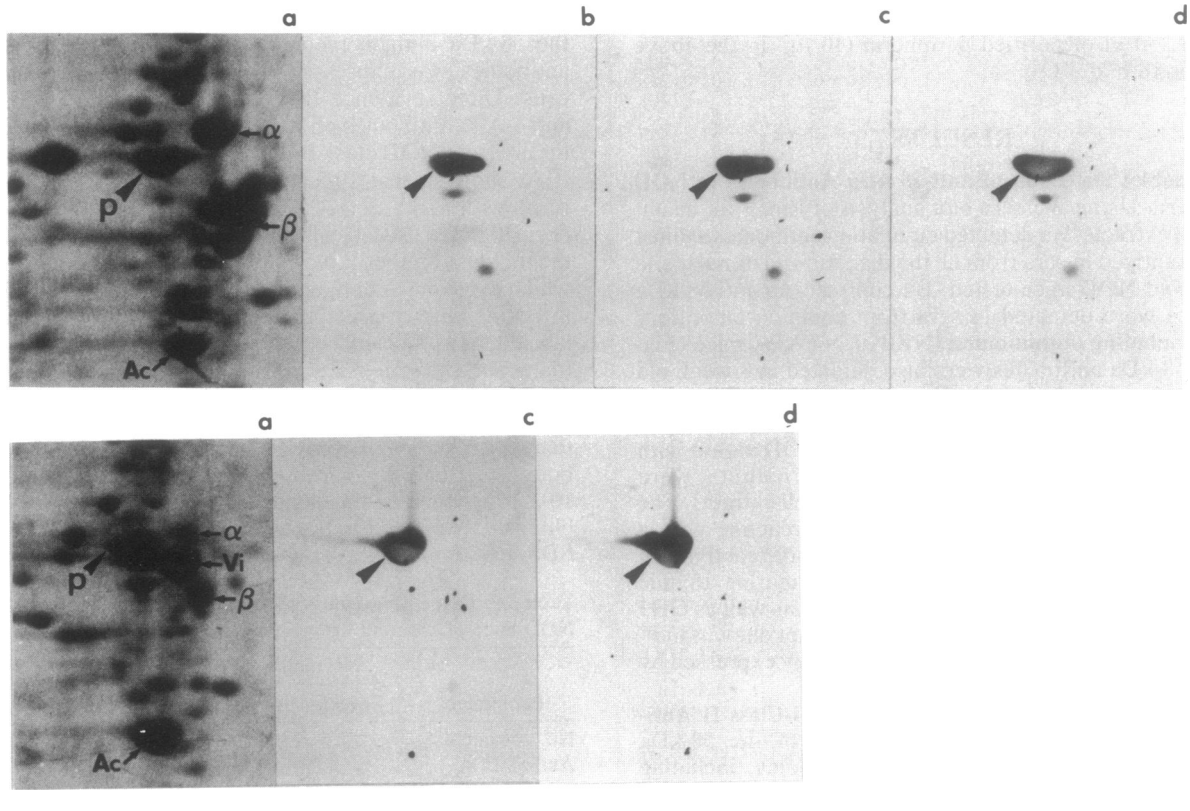

FIG. 2. Western blots of two-dimensional SDS/PAGE extracts of Rin5F (*Upper*) and NIE115 mouse neuroblastoma (*Lower*) cells. Cell extracts blotted on nitrocellulose filters were tested for reactivity with diabetic NOD mouse serum (*b*), monoclonal antibody 10-2-16 (*c*), or monoclonal antibody 72-2 (*d*). (*a*) Coomassie blue-stained gel. Ac, actin; α-β, tubulin; Vi, vimentin; P, peripherin.

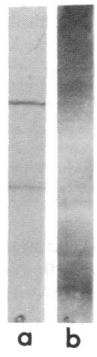

FIG. 3. Absorption experiment. Monoclonal antibody 72-2 was tested on Rin5F cell extracts as in Fig. 1 (lane a) or after absorption with purified murine peripherin (lane b).

lating mouse autoantibodies and the 72-2 NOD monoclonal antibody were shown to recognize the 58-kDa antigen both in the native form on live cells by RIA and in the denatured form on islet cell extracts by Western blot analysis. The presence of anti-58-kDa autoantibodies early in the lifespan of NOD mice—i.e., during the development of insulitis and prior to initial β-cell destruction (30)—could signify that their production is related to the early disease process.

Apart from insulin-secreting cells, the 58-kDa antigen was only found to be expressed by neuroblastoma cells. The concomitant expression of the autoantigen recognized by NOD mouse autoantibodies by insulin-secreting and neuroblastoma cells, together with the results of absorption experiments, identified the 58-kDa autoantigen as peripherin, an intermediate filament protein previously characterized in well-defined neuronal populations (31, 32). Peripherin has been cloned and sequenced from the NIE 115 neuroblastoma cell λgt11 library and comprises three closely related products originating from alternative splicing of a single gene (32). The islet antigen recognized by NOD and anti-class II antibodies appears to be the major 58-kDa isoform. The 58-kDa isoform expressed by islet and neuronal-related cells was shown to have the same mobility by two-dimensional gel electrophoresis using isoelectric focusing and SDS/PAGE. Considering these similarities, it is likely that peripherin is expressed in the cytoplasmic space in islet cells. In neuroblastoma cells, peripherin attaches to both the plasma and the nuclear membrane to participate in the intermediate filament network within the cytoplasmic space. Nuclear lamin B has been demonstrated as a specific attachment site for peripherin intermediate filaments (33). Interestingly, concomitant expression of other antigens on pancreatic islet-related cells and neuronal cells has previously been reported (16, 34, 35). In particular, the dual distribution of peripherin is reminiscent of that of a recently identified 64-kDa antigen, glutamic acid decarboxylase (16). The expression of neural structural markers by Langerhans islet cells may suggest a lineage relationship with ectoderm-derived cells.

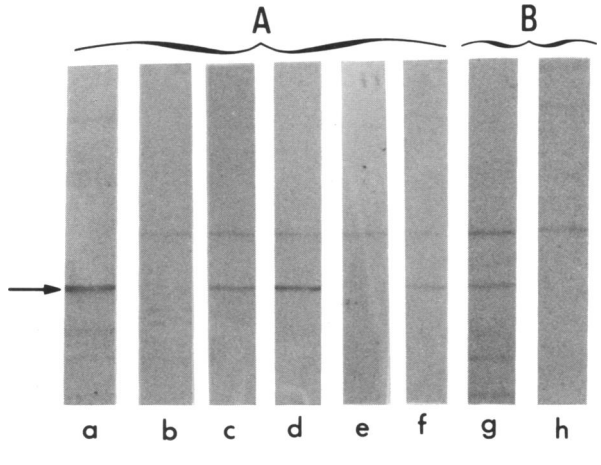

FIG. 4. Cross-blocking experiments. Rin5F Western blots were incubated for 30 min with PBS (lanes a and g), 72-2 (lane b), 10-2-16 (lanes c and h), MKD6 (lane d), NOD (lane e), or DBA/2 (lane f) sera. Then they were washed and incubated with biotinylated 72-2 (*A*) or with biotinylated 10-2-16 (*B*) for 10 min. The arrow indicates the 58-kDa bands.

The 58-kDa autoantigen that we detected on islet cells was also recognized by two mouse anti-class II monoclonal antibodies. Both antibodies react with peripherin and spleen cells expressing I-A^{nod} (16) and share the Ia17 specificity characteristic of $A\beta^{k}$ (36). This showed the existence of a cross-reactive epitope shared by I-A^{nod} class II antigens and an islet autoantigen eliciting specific autoantibodies in the NOD mouse. The epitope is a polymorphic site of the I-A antigen A_{β} chain characteristic of the NOD strain and is distinct from that recognized by NOD mouse autoantibodies on peripherin. Cross-reactivity between class II antigens and ubiquitous antigens such as myosin, actin, spectrin, and tubulin has previously been reported (37, 38). It remains to be determined whether the 58-kDa antigen cross-reactivity with class II NOD antigens is involved in triggering the autoimmune reaction that leads to islet β-cell destruction. The mechanisms by which self-tolerance of extrathymic autoantigens is established are poorly understood. At all events, the existence of a cross-reactive epitope between islet cell and class II antigens may favor the expansion of anti-islet autoreactive T-cell clones within the thymus. It is unlikely that such cross-reactivity influences immune function mediated by peripheral T cells, although defective T-cell regulation has been reported in the NOD mouse (39).

Further studies are required to evaluate the role of peripherin in the development of diabetes. Autoreactive T-cell lines and clones have recently been obtained in the NOD mouse model (40–42). Whether or not peripherin induces T-cell sensitization needs to be assessed in studies aimed at generating peripherin-specific T-cell clones in NOD mice, as recently reported for heat shock proteins (42). Peripherin is expressed by neuronal populations of various lineages during murine development, and the analysis of its expression by islet cells will help in understanding its role in the induction of autoimmunity. The cloning and sequencing of peripherin (32) should help to identify the factors controlling its expression, as well as immunologically relevant constitutive peptides.

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