

Islet-infiltrating B-Cells in Nonobese Diabetic Mice Predominantly Target Nervous System Elements

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B-cells accumulate in pancreatic islets during the autoimmune response that precedes the onset of type 1 diabetes. However, the role and antigenic specificity of these cells remain a mystery. To elucidate the antigenic repertoire of islet-infiltrating B-cells in type 1 diabetes, we generated hybridoma cell lines of islet-infiltrating B-cells from nonobese diabetic (NOD) mice and NOD mice expressing a diabetogenic T-cell receptor (8.3-NOD). Surprisingly, characterization of the tissue specificity of the antibodies secreted by these cells revealed that a predominant fraction of these hybridomas produce antibodies specific for the pancreatic nervous system. Similar results were obtained with B-cell hybridomas derived from mild insulinitic lesions of diabetes-resistant (NOD × NOR)F1 and 8.3-(NOD × NOR)F1 mice. Immunoglobulin class analyses further indicated that most islet-derived hybridomas had arisen from B-cells that had undergone immunoglobulin class switch recombination, suggesting that islet-associated B-cells are involved in active, T-helper-driven immune responses against local antigenic targets. This is the first evidence showing the existence of a predominant active B-cell response in situ against pancreatic nervous system elements in diabetogenesis. Our data are consistent with the idea that this B-cell response precedes the progression of insulinitis to overt diabetes, thus strongly supporting the idea that pancreatic nervous system elements are early targets in type 1 diabetes. *Diabetes* 54:69–77, 2005

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FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; RAG, recombination-activating gene; TRITC, tetramethyl rhodamine isothiocyanate. © 2005 by the American Diabetes Association.

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Type 1 diabetes is a complex autoimmune disease characterized by selective destruction of pancreatic β -cells by the patient's own immune system (1). Currently, one of the best animal models of type 1 diabetes is the nonobese diabetic (NOD) mouse. NOD mice spontaneously develop a form of diabetes that closely resembles human type 1 diabetes (2). Studies with this animal model have shown that islet-infiltrating T-cells are major effectors of β -cell damage in type 1 diabetes and that these T-cells progressively destroy islet β -cells over a long, protracted period of insulinitis (3,4). The antigenic repertoire of β -cell-reactive T-cells in NOD mice involves a wide range of β -cell autoantigens, including GAD, insulin, carboxypeptidase H, HSP65, and the recently described islet-specific glucose-6-phosphatase catalytic subunit-related protein, among others (4,5). Although the main targets of the diabetogenic autoimmune attack are β -cells, other islet cell populations may also be affected by the local autoimmune response (3). Previous studies, for example, have suggested that insulinitis in NOD mice is accompanied by a T-cell response directed against the autonomous nervous system that envelops pancreatic islets (6,7). In fact, peripheral T-cell reactivity against neuroantigens such as glial fibrillary acidic protein (GFAP) or peripherin has been described in NOD mice (7,8).

Diabetogenesis, however, not only involves the recruitment of autoreactive T-cells but also the recruitment of other cellular elements of the immune system. Among these, B-cells are thought to play a critical role in the initiation and/or progression of type 1 diabetes, at least in NOD mice (9–11). Although the mechanisms by which B-cells contribute to diabetes development remain poorly understood, it has been proposed that they do so by capturing β -cell autoantigens via cell surface autoreactive immunoglobulins and by presenting these antigens to autoreactive T-cells (12–14). Several lines of evidence support this notion. First, type 1 diabetes development in both human and NOD mice is associated with the presence of circulating autoantibodies against several β -cell autoantigens (8,15). Second, transmission of β -cell-reactive autoantibodies from pregnant NOD females to fetuses during pregnancy contributes to the progression of diabetes in the offspring (16).

Our current appreciation of the antigenic repertoire of autoreactive B-cells in type 1 diabetes almost exclusively stems from studies of the antigenic specificity of circulat-

ing islet-reactive autoantibodies (8) and from analyses of the antigenic specificity of peripheral B-cell hybridomas (from human blood [17–19] or rodent spleen [20–25]). In general, these studies have shown the existence, in both pre- and acutely diabetic individuals, of an extensive peripheral B-cell response against a wide range of β -cell autoantigens, including GAD, insulinoma-associated protein 2, insulin, carboxypeptidase H, ICA69, and ganglioside GM2–1.

The present study aimed at elucidating the antigenic specificity and repertoire of islet-infiltrating B-cells in both diabetes-prone and diabetes-resistant (but insulinitis-prone) mice. Our data show that, contrary to our expectations, the antigenic specificity of islet-infiltrating B-cells in pre-diabetes is predominantly directed against nervous system elements of the islet. Therefore, by using a different scientific approach, our results lend strong support to the hypothesis that an autoimmune attack to pancreatic nervous system elements is an early event in the natural history of type 1 diabetes (6,7,26).

RESEARCH DESIGN AND METHODS

NOD and NOR (27) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained by brother-sister mating under specific pathogen-free conditions according to the European Laws and Directives European Community concerning the protection of animals for experimentation. 8.3-NOD and NOD.RAG (recombination-activating gene)-2^{-/-} mice have been described elsewhere (28). 8.3-NOD mice are transgenic mice that express the T-cell receptor of a diabetogenic CD8⁺ T-cell clone (NYS.3), specific for the β -cell autoantigen islet-specific glucose-6-phosphatase catalytic subunit-related protein (5). Importantly, this T-cell receptor is representative of those expressed by a predominant population of pathogenic CD8⁺ T-cells that infiltrate islets in pre-diabetic NOD mice. As a result, 8.3-NOD mice develop an accelerated form of autoimmune diabetes (28). NOD.RAG-2^{-/-} mice are RAG-2-deficient NOD mice and develop neither diabetes nor insulinitis. (NOD \times NOR)F1 and 8.3-(NOD \times NOR)F1 mice (referred to as F1 and 8.3-F1 mice, respectively) were generated by crossing NOR mice with NOD or 8.3-NOD mice. The majority of F1 females (60%) only develop a nondestructive but severe form of insulinitis, and only ~33% develop overt diabetes (29). The incidence of disease in 8.3-F1 mice is similar to that observed in nontransgenic (NOD \times NOR) F1 mice (30). Therefore, F1 and 8.3-F1 mice are useful models to study mechanisms underlying the progression of insulinitis to overt diabetes.

Twenty-week-old female Sprague Dawley rats were obtained from Charles River Laboratories (St. Germain sur l'Arbresle, France). The pancreas of each animal was frozen in liquid nitrogen and kept at -80°C until used for immunohistological staining.

Human pancreatic sections for immunohistological studies were obtained from a cadaveric organ donor (32-year-old man; blood type O). The pancreas was extracted 30 min after heart arrest with the family's consent according to the guidelines of our institution's ethics committee and the principles included in the Declaration of Helsinki.

Antibodies and cell lines. The polyclonal antibodies rabbit anti-glucagon, rabbit anti-somatostatin, rabbit anti-cow GFAP, and IgG1 mouse monoclonal antibody anti-neurofilament 200 were obtained from Dako (Carpinteria, CA). The polyclonal antibody guinea pig anti-pig insulin came from Sigma (St. Louis, MO). The polyclonal antibody goat anti-peripherin was from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal antibodies goat anti-mouse Ig horseradish peroxidase and biotin-conjugated mouse anti-mouse IgG2a^b (Igh-1b) monoclonal antibody were purchased from BD Biosciences (Erembodegem, Belgium). The polyclonal antibody rabbit anti-goat Ig-Alexa Fluor 488 was obtained from Molecular Probes (Leiden, the Netherlands). The polyclonal antibodies goat anti-mouse IgM+G+A fluorescein isothiocyanate (FITC), goat anti-mouse IgG1 labeled with FITC, goat anti-rabbit tetramethyl rhodamine isothiocyanate (TRITC), goat anti-mouse Ig TRITC, and streptavidin FITC were obtained from Southern Biotechnology Associates (Birmingham, AL). The polyclonal antibody goat anti-guinea pig IgG TRITC was from ICN Biomedicals (Eschwege, Germany), and, finally, cell lines NS-1 and NIE-115 were obtained from the American *Type Culture* Collection.

Hybridoma production. Pancreatic islets were isolated as described elsewhere (31) and cultured in complete media (RPMI-1640 media containing 10%

heat-inactivated fetal bovine serum [Life technologies, Grand Island, NY], 50 units/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin, 200 mmol/l glutamine, 1 mmol/l sodium pyruvate, and 50 $\mu\text{mol}/\text{l}$ 2-ME [Sigma-Aldrich]) in the presence of 10 $\mu\text{g}/\text{ml}$ lipopolysaccharide (Sigma-Aldrich) to ensure optimal B-cell conditions for hybridoma production. Twelve hours later, mononuclear cells migrating into the culture media were fused with the NS-1 myeloma cell line at a 1:2 ratio, using polyethylene glycol. Thirty-three fusion procedures were conducted using islet-infiltrating mononuclear cells from 67 NOD and 55 F1 mice aged between 8 and 14 weeks, 35 8.3-NOD and 35 8.3-F1 mice aged between 6 and 10 weeks, 6 acutely diabetic NOD, and 5 diabetic 8.3-NOD mice. The number of mononuclear cells used in each fusion was between 2×10^5 and 2.6×10^6 and came from pools of pancreatic islets isolated from two to five mice. Fused cell lines were cultured in hypoxanthine-, aminopterin-, and thymidine-selective (HAT) media for 2 weeks; then, the growing hybridomas were screened for antibody production by enzyme-linked immunosorbent assay and pancreas specificity by immunofluorescence staining. Hybridomas positive for antibody production were cloned twice consecutively by limiting dilution. Positive clones were expanded and culture supernatants collected for further immunohistological studies.

Hybridoma screening. Hybridoma supernatants were adsorbed overnight at 4°C in a carbonate buffer on Nunc-immuno F96 Maxisorp plates (Nalge Nunc International). Buffered saline 0.5% BSA was used to block any residual binding capacity of the plates. Wells were washed with PBS-Tween and then incubated with goat anti-mouse Ig horseradish peroxidase for 60 min at room temperature. The antigen-antibody reaction was developed with the chromogen o-phenyldiamine (Dako). Absorbance was read using a 450-nm filter, with a reference wavelength of 620 nm.

Immunofluorescence staining. Cryosections (5 μm) of pancreata from NOD.RAG-2^{-/-} mice, Sprague Dawley rats, and human cadaveric organ donors were air dried for 30 min, incubated at 4°C overnight with 30 μl of each hybridoma supernatant, washed, and then incubated with the secondary antibody (goat anti-mouse IgM+G+A FITC or goat anti-mouse Ig TRITC) at 4°C for 45 min. The concentration dependence of the monoclonal antibody staining was evaluated as described above using serial dilutions of either the hybridoma supernatants (5–100 times dilutions) or from purified monoclonal antibodies (hybridomas H184, H116, and H173; dilutions from 0.01 to 0.00001 mg/ml). For most of the nonpurified monoclonal antibodies, immunofluorescence staining was lost between 10 and 50 times supernatant dilution, whereas for those purified monoclonal antibodies, immunofluorescence staining was kept up to 0.001–0.0001 mg/ml. Double-staining studies were conducted as described above, with two more incubation steps with rabbit anti-glucagon, rabbit anti-somatostatin, rabbit anti-GFAP, IgG1 mouse monoclonal antibody anti-neurofilament 200, goat anti-peripherin, or guinea pig anti-pig insulin and subsequently with a goat anti-rabbit TRITC, goat anti-mouse IgG1 FITC, rabbit anti-goat Ig-Alexa Fluor 488, or goat anti-guinea pig IgG TRITC, respectively. Cryosections from parotid, mixed salivary gland, bronchial gland, thyroid, brain, stomach, and kidney from NOD.RAG-2^{-/-} mice were used to establish reactivity to other tissues. The presence of monoclonal antibodies to nuclear antigens and native DNA was confirmed using a commercial (Kallestad) substrate of the Hep-2 cell line and *Crithidia luciliae*, respectively. Intracellular staining of the neuroblastoma NIE-115 cell line was done as follows. A single cell suspension of the cell line was cultured overnight on sterile coverslips into 24-well tissue culture plates. On the following day, the coverslips were recovered and washed twice with PBS; then, the cells were fixed in 1% paraformaldehyde at room temperature for 5 min, permeabilized with 0.3% Triton-X in PBS, and stained as described above with 30 μl hybridoma supernatant for 1 h, followed by the secondary antibody (goat anti-mouse IgM+G+A FITC) at 4°C for 45 min. The preparations were analyzed with a confocal microscopy (Axioskop2, ref. no. 800779; Zeiss).

Detection of antibody isotype. Antibody isotypes were determined using a mouse monoclonal antibody isotyping kit (Serotec) following the manufacturer's instructions. The product of the NOD mouse Igh-1 locus is IgG2c, rather than IgG2a (32); the IgG2c isotype is not included in the kit. Thus, for antibody-secreting hybridomas that were false-positive for IgG2a (due to cross-reactivity of the anti-mouse IgG2a antibody with IgG2c), the true isotypic specificity of the antibody was defined as follows: 1) by analyzing the cDNA sequence of the Igh CH3 region, which is the most polymorphic region of the Igh-1 gene locus, thus allowing us to easily differentiate between the two isotypes (the primers used for cDNA amplification were sense 5'-GTA-TAT-GTC-TTG-CCT-CCA-CCA-3'; antisense 5'-TAA-GAA-CCA-TCA-GAG-TCC-AGG-3'), and 2) by immunohistochemistry using cryosections of NOD.RAG-2^{-/-} mice pancreata using biotin-conjugated mouse anti-mouse IgG2a^b (Igh-1b) monoclonal antibody as secondary antibody and then streptavidin FITC.

TABLE 1
Hybridoma production

	Number of fusion procedures	Total number of hybridomas obtained	Antibody-secreting hybridomas
NOD	8	49	10
NOD type 1			
diabetic	3	5	2
8.3-NOD	5	46	5
8.3-NOD type 1			
diabetic	5	105	13
F1	6	52	17
8.3-F1	6	95	27
Total	33	352	74

RESULTS

Hybridoma production. From 33 independent fusion procedures, we obtained a total of 352 hybridomas, 74 of which were antibody-secreting hybridomas (Table 1). The apparently low percentage of antibody-secreting hybridomas may be caused by the own characteristics of mononuclear islet infiltrates (such as B-cell percentage or their functionality and degree of activation) or to differences in the presence of some islet environmental factors (such as proinflammatory cytokines) affecting cell status. Moreover, we cannot rule out the possibility that the long and aggressive procedure necessary to obtain islet-infiltrating B-cells may also affect hybridoma production.

A predominant number of islet-derived antibody-secreting hybridomas originate from islet-infiltrating B-cells with specificity for pancreatic nervous system elements. To elucidate the antigenic repertoire of islet-associated B-cells in NOD mice, we characterized the tissue and cell specificity of the corresponding hybridomas. We obtained a total of 54 hybridomas from 11 independent fusion procedures. Twelve of these hybridomas secreted antibodies (Table 1). Immunofluorescence analysis using monoclonal antibodies secreted by these hybridomas on NOD.RAG-2^{-/-} pancreas cryosections revealed that five of these antibody-secreting hybridomas recognized pancreatic tissue components other than β -cells (Fig. 1A and Table 2), whereas the remaining seven were negative for pancreatic tissue. Two of these five hybridomas produced monoclonal antibodies that reacted to exocrine tissue or extracellular matrix (herein referred to as exocrine and extracellular connective patterns, respectively). Interestingly, the remaining three antibody-secreting hybridomas (three of five) produced antibodies that stained the same pancreatic tissue elements that reacted with the anti-peripherin, anti-neurofilament 200, and/or anti-GFAP antibodies alone (Fig. 1C and Table 2). GFAP is an intermediate filament present in neuroglial cells of the peripheral nervous system (Schwann cells, satellite cells, and enteric glial cells). In fact, these three antibodies also stained the mouse neuroblastoma cell line N1E-115 (data not shown), thus indicating specificity for nervous system components. Two representative nervous system staining patterns were observed: one (referred to as nervous system pattern G) matched almost completely the peripherin, neurofilament 200, and GFAP staining patterns. The other (referred to as nervous system pattern

H) displayed a fibrillar-like staining pattern that colocalized only partially with GFAP, but neither colocalized with peripherin nor neurofilament 200.

To further substantiate these results, we characterized the specificity of B-cell hybridomas derived from islets of 8.3 T-cell receptor transgenic NOD mice (28), in which damage of peri-islet Schwann cells has been recently shown to precede massive β -cell destruction (7). We obtained 151 hybridomas from 10 independent fusion procedures. Twelve of 18 monoclonal antibody-secreting hybridomas (Table 2) secreted antibodies that reacted to pancreatic tissue components, whereas the remaining were negative for pancreas. Interestingly, 4 of the 12 hybridomas produced monoclonal antibodies that reacted with an islet antigen that colocalized with peripherin, neurofilament 200, and/or GFAP, again suggesting reactivity against pancreatic nervous system components (Fig. 1A and Table 2). Only 1 of the 12 antibody-secreting hybridomas produced antibodies that exhibited an islet staining pattern (referred to as the mixed pattern) that could not be reproduced with anti-glucagon, anti-somatostatin, anti-GFAP, or anti-insulin antibodies and that was characterized by staining of only a few scattered positive cells inside islets and in exocrine pancreatic tissue. The remaining seven hybridomas (7 of 12) produced monoclonal antibodies that recognized exocrine tissue, cell nucleus, or extracellular matrix (herein referred to as exocrine, nuclear, and extracellular connective patterns, respectively). The specificity of the hybridoma with nuclear pattern (anti-DNA) was confirmed by staining the Hep-2 cell line and *Crithidia luciliae* (Fig. 1A, *k* and *n*). **The antigenic specificity of B-cells recruited to pancreatic tissue in non-diabetes-prone mice is also skewed toward nervous system components of the pancreas.** We next set out to investigate whether the predominant intraislet B-cell response against nervous system elements that we saw in NOD and 8.3-NOD mice was secondary to islet β -cell destruction. This was done by characterizing the antigenic specificity of antibody-secreting B-cell hybridomas derived from infiltrated islets of diabetes-resistant (but insulinitis-prone) (NOD \times NOR)F1 and 8.3-(NOD \times NOR)F1 mice. As shown in Fig. 1A and Table 2, the largest proportion of antibody-secreting hybridomas from both types of mice produced monoclonal antibodies that recognized nervous system antigens. In (NOD \times NOR)F1 mice, 11 of 17 antibody-secreting hybridomas displayed reactivity for pancreatic tissue components and 9 of these 11 hybridomas produced antibodies that colocalized with peripherin, neurofilament 200, and/or GFAP. One of the remaining two antibody-secreting hybridomas recognized pancreatic islets. However, the corresponding monoclonal antibody displayed an islet-staining pattern (Fig. 1A, *B*, and *C* and Table 2) that overlapped with that obtained using both anti-insulin and anti-peripherin, anti-neurofilament 200, or anti-GFAP antisera. We refer to this staining pattern as the neuroendocrine pattern. The remaining hybridoma produced a monoclonal antibody that was specific for a nuclear antigen (Fig. 1A, *l*). With regards to 8.3-(NOD \times NOR)F1 mice, 24 of 27 antibody-secreting hybridomas produced monoclonal antibodies that reacted to pancreatic tissue components, whereas 3 were negative for pancreas. Notably, 12

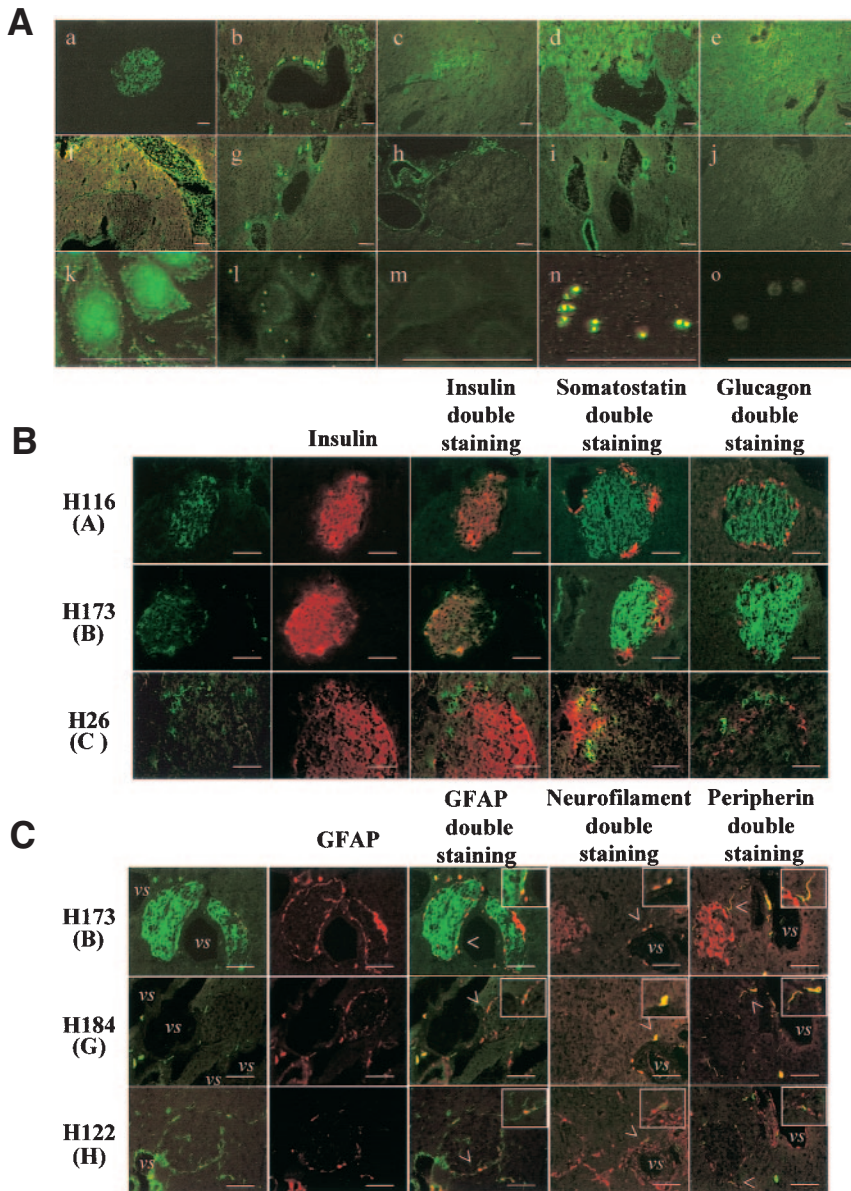


FIG. 1. Antigenic specificity of antibody-secreting hybridomas. **A:** Culture supernatants from antibody-secreting hybridomas were incubated on cryosections (5 μm) from the pancreata of NOD, RAG-2^{-/-} mice (*a–j*), Hep-2 cell line (*k–m*), or *Crithidia luciliae* (*n* and *o*). The secondary antibody was a goat anti-mouse IgM+G+A labeled with FITC. The antibody-secreting hybridomas were grouped according to their staining patterns as follows: *a*, inraislet (representative hybridoma H116); *b*, neuroendocrine (hybridoma H173); *c*, mixed (hybridoma H26); *d–f*, exocrine (representative hybridomas H226, H1, and H209, respectively); *g* and *h*, nervous system (representative hybridomas H184, and H122, respectively); *i*, extracellular connective (representative hybridoma H196); *j*, tissue negative control; *k* and *n*, nuclear anti-DNA on Hep-2 and *Crithidia luciliae*, respectively (hybridoma H149); *l*, a few nuclear dots staining the nucleus of Hep-2 (hybridoma H301); *m* and *o*, negative control of Hep-2 and *Crithidia luciliae*, respectively. **B** and **C:** Double immunostaining of the monoclonal antibodies from antibody-secreting hybridomas (green or red) with anti-insulin (red), anti-glucagon (red), anti-somatostatin (red), anti-GFAP (red), anti-neurofilament 200 (green), or anti-peripherin (green). Staining patterns: inraislet (hybridoma H116), neuroendocrine (hybridoma H173), mixed (hybridoma H26), and nervous system (hybridomas H184 and H122). *vs* refers to blood vessel or pancreatic duct. The arrow indicates the enlargement area (small square on the upper right corner of each picture). Bar = 50 μm .

of these 24 monoclonal antibodies recognized nervous system elements, and only 3 recognized pancreatic islets. The latter monoclonal antibodies displayed a staining pattern that mimicked the one obtained with anti-insulin antibodies, suggesting specificity for antigenic determinants selectively expressed in islet β -cells (Fig. 1B and Table 2). We refer to this pattern as the β -cell pattern. The remaining nine antibody-secreting hybridomas produced monoclonal antibodies that had affinity for exocrine tissue ($n = 8$) or extracellular matrix ($n = 1$). Thus, when taken together, these results indicated that the predominant anti-nervous tissue element B-cell response that we saw in NOD mice also occurs in mice in which islet β -cell destruction is limited. The data therefore suggest that predominant recruitment of nervous tissue-specific B-cells to pancreatic islets in diabetogenesis is dissociated from (and possibly not affected by) islet β -cell destruction. **Most islet-associated, antibody-secreting hybridomas specific for pancreatic islets and nervous system derive from B-cells that have undergone**

immunoglobulin class switch recombination. To establish whether antibody-secreting hybridomas arose from islet-associated B-cells involved in an active immune response to pancreatic tissue, we determined the isotype of the secreted monoclonal antibodies. Of the 52 antibody-secreting hybridomas that were tested, 20 produced IgM monoclonal antibodies, 3 were IgG1, 9 were IgG2c, 19 were IgG2b, and 1 was IgA (Table 2). All monoclonal antibodies used the κ light chain. For antibody-secreting hybridomas restricted to exocrine, nuclear, and extracellular connective determinants, IgM was the prevailing isotype, suggesting that they originated from B-cells producing polyreactive natural antibodies. Interestingly, most islet- and nervous tissue-specific hybridomas secreted IgG2b, IgG2c, and IgG1 antibodies (in this order of frequency), indicating that they derived from precursors that had undergone class switch recombination and, probably, affinity maturation.

The antigenic specificity of islet-infiltrating B-cells is not restricted to the pancreas. To explore whether

antigens recognized by our monoclonal antibodies were restricted to the pancreas, we investigated antibody reactivity with other tissues. Cryosections of parotid gland, mixed salivary gland, bronchial gland, thyroid, brain, stomach, and kidney from NOD.RAG-2^{-/-} mice were stained using one representative monoclonal antibody for each staining pattern (Fig. 2). Monoclonal antibodies with exocrine or extracellular connective staining patterns extensively reacted with all the tested tissues. Likewise, monoclonal antibodies displaying a nervous system staining pattern reacted to most tissues analyzed, indicating that they are directed against ubiquitous antigen(s) of the nervous system. Significantly, monoclonal antibodies with a β -cell-specific staining pattern cross-reacted exclusively with few cells present in the stomach and kidney.

Then, we analyzed whether those antibody-secreting hybridomas negative for pancreas tissue could react with other NOD.RAG-2^{-/-} mouse organs. As shown in Table 3, 7 of a total of 22 pancreas negative antibody-secreting hybridomas displayed reactivity to several other mouse tissues. Interestingly, three of seven reacted with brain and other tissue specificities, whereas two of seven solely targeted nervous system elements.

Cross-reactivity with rat and human species. Once we had established that reactivity of representative monoclonal antibodies was not restricted to the pancreas, we set out to determine whether they could also recognize similar antigens in rats and humans. To this end, we analyzed the reactivity of these monoclonal antibodies on cryosections of human and rat pancreas. As shown in Fig. 3, most antibodies cross-reacted with antigens expressed in rat and human pancreata, therefore indicating that the intrapancreatic B-cell response that takes place during the initiation and/or progression of type 1 diabetes is directed against antigenic determinants that are conserved across species.

DISCUSSION

Like their counterparts, the T-cells, B-cells migrate to pancreatic islets during the development of type 1 diabetes. Once in the pancreas, T-cells kill β -cells, leading to both insulin deficiency and onset of the clinical manifestations of diabetes. The role(s) that islet-associated B-cells play in the disease process, however, remain(s) largely unknown. Several lines of evidence have suggested that B-cells are key to the development of spontaneous autoimmune diabetes. Depletion of this cell population in wild-type NOD (10) or NOD.*Ig μ ^{null}* mice (9,11,33) results in type 1 diabetes resistance and/or in a delay in the age at onset of the disease. Conventional wisdom would argue that B-cells contribute to diabetogenesis by presenting autoantigens (presumably shed into the milieu by a prior insult and subsequently captured by cell surface immunoglobulins) to major histocompatibility complex class II-restricted autoreactive CD4⁺ T-cells (12,13). A sine qua non condition for this hypothetical ability of islet-associated B-cells to function as professional antigen-presenting cells in type 1 diabetes, however, is that they must express immunoglobulin molecules specific for the same autoantigens they present to autoreactive T-cells (34).

Others have previously approached the analysis of the B-cell antigenic repertoire in type 1 diabetes by deriving

TABLE 2
Staining patterns and isotypes produced by the antibody-secreting hybridomas in NOD.RAG-2^{-/-} mouse pancreas

Staining pattern	Representative hybridoma	IgM				IgG1				IgG2c				IgG2b				IgA				TOTAL	
		NOD	8.3-NOD	F1	8.3-F1	NOD	8.3-NOD	F1	8.3-F1	NOD	8.3-NOD	F1	8.3-F1	NOD	8.3-NOD	F1	8.3-F1	NOD	8.3-NOD	F1	8.3-F1		
Intraislet Neuro-endocrine	(a) H116																						3
Mixed Exocrine	(b) H173																						1
	(c) H26		1																				1
	(d) H226		4		2																		7
	(e) H1		1		1																		4
Neuronal	(f) H209	1			3																		3
	(g) H184																						3
	(h) H122	1	1	1																			25
																							3
Extracellular connective Nuclear	(i) H196																						3
	(j) H149		1		1																		1
	(k) H301			1																			1
																							1
Pancreas negative		5	3	5	2	2	3	1	1	1	1	1	4	4	4	2	2	5	10	1			22
	Total	7	12	7	9	2	3	1	4	4	4	2	2	5	10	1							74

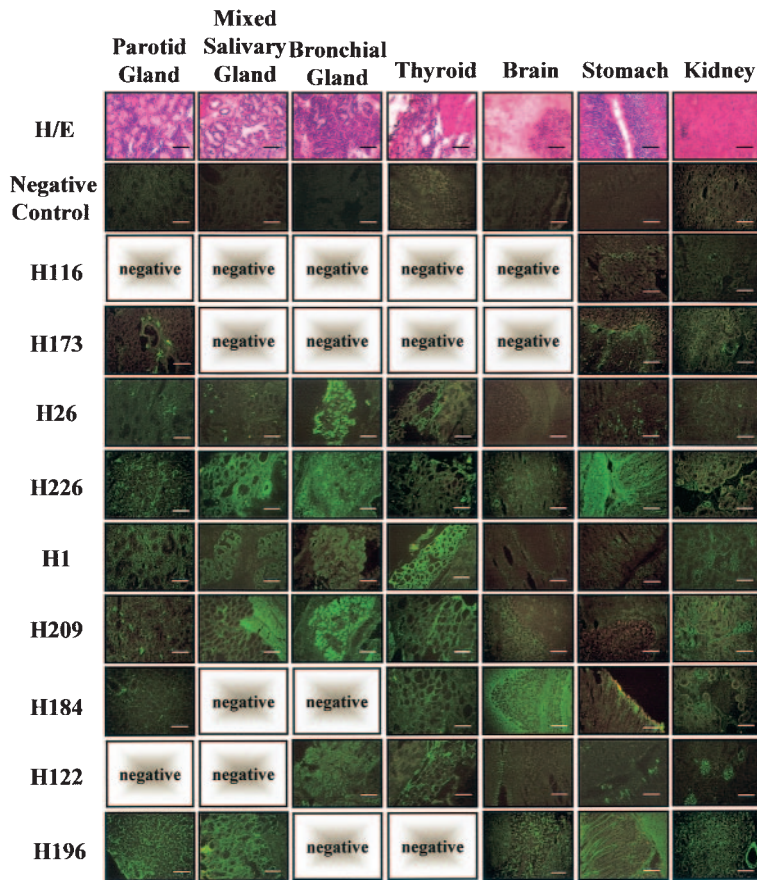


FIG. 2. Reactivity of monoclonal antibodies produced by the antibody-secreting hybridomas in several NOD.RAG-2^{-/-} mouse organs. Culture supernatants from a group of antibody-secreting hybridomas representative of each pancreas staining pattern were incubated on cryosections (5 μm) from parotid gland, mucous salivary gland, bronchial gland, thyroid, brain, stomach, and kidney from NOD.RAG-2^{-/-} mice. The secondary antibody was a goat anti-mouse IgM+G+A labeled with FITC. Bar = 50 μm.

antibody-secreting hybridomas from human peripheral blood B-cells (17–19) and NOD mouse or BB rat spleen cells (20–25). All of these studies suggested that islet cell-specific B-cells were relatively frequent in the circulation or in the spleen. In one study (23), for example, as

many as 35 of ~4,000 spleen-derived hybridomas were islet specific. Unexpectedly, here we have found that a large fraction of all islet-associated B-cells in NOD mice produce antibodies that recognize antigens that are not β-cell specific. In fact, most of these antibodies recognize

TABLE 3

Tissue specificity of the monoclonal antibodies produced by the 22 pancreas negative antibody-secreting hybridomas in several NOD.RAG-2^{-/-} mouse organs

Hybridoma	Isotype	Source	Parotid	Mixed salivary gland	Bronchial gland	Thyroid	Brain	Stomach	Kidney
221B	IgM κ	NOD	–	+	+	–	+	–	–
285	IgG1 κ	NOD	–	–	–	–	–	–	–
279	IgM κ	NOD	–	–	–	–	–	–	–
253	IgG1 κ	NOD	–	–	–	–	–	–	–
243	IgM κ	NOD	–	–	–	–	–	–	–
221	IgM κ	NOD	–	–	–	–	–	–	–
212	IgM κ	NOD	–	–	–	–	–	–	–
56	IgG1 κ	8.3-NOD	–	–	–	–	–	–	–
112	IgM κ	8.3-NOD	–	–	–	–	+	–	–
74	IgG1 κ	8.3-NOD	–	–	–	–	+	–	–
98	IgM κ	8.3-NOD	–	–	–	–	–	–	–
147	IgM κ	8.3-NOD	–	–	–	–	–	–	–
156	IgG1 κ	8.3-NOD	–	–	–	–	–	–	–
205	IgM κ	8.3-F1	–	–	–	–	–	–	–
270	IgG1 κ	8.3-F1	–	–	–	–	–	–	–
117	IgM κ	8.3-F1	–	–	–	–	–	–	–
192	IgM κ	(NODXNOR)F1	–	–	–	–	–	–	–
298	IgG1 κ	(NODXNOR)F1	–	–	–	–	–	–	–
183-39	IgM κ	(NODXNOR)F1	–	–	+	–	–	–	+
171D	IgM κ	(NODXNOR)F1	–	+	+	–	+	–	+
172B	IgM κ	(NODXNOR)F1	–	–	–	–	+	–	–
84C	IgM κ	(NODXNOR)F1	–	+	+	–	–	–	–

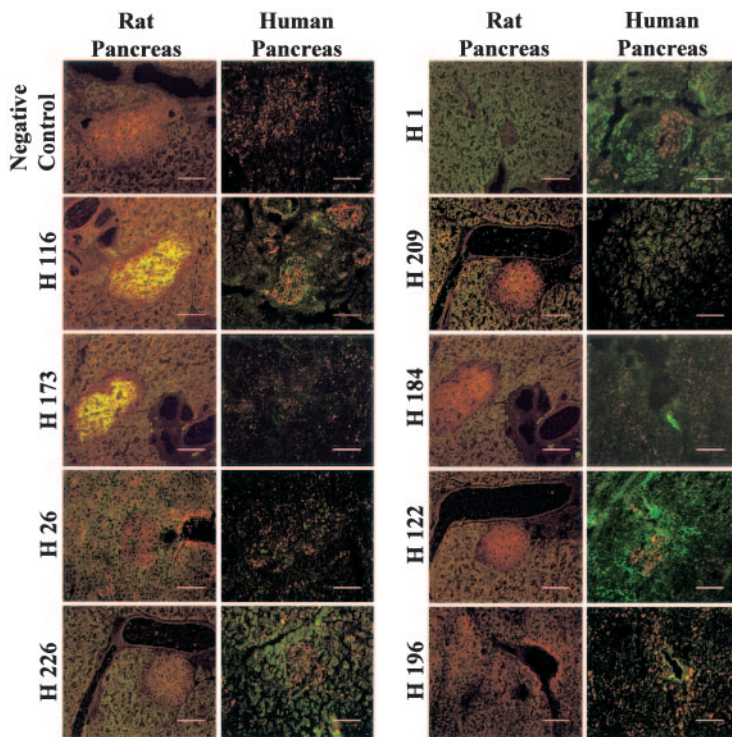


FIG. 3. Cross-reactivity of monoclonal antibodies in human and rat pancreas. Culture supernatants from a the same representative group of antibody-secreting hybridomas were incubated on cryosections ($5\ \mu\text{m}$) of human and rat pancreas. The secondary antibody was a goat anti-mouse IgM+G+A labeled with FITC. Cross-reactivity to rat and human pancreata is detected for all the monoclonal antibodies except for the monoclonal antibodies H226 and H196 on neither human nor rat and the monoclonal antibodies H173 and H209 on human pancreas. Bar = $50\ \mu\text{m}$.

non-islet-specific antigens shared by certain intra- and extrapancreatic nervous system elements. We also show that the antigenic repertoire of these islet-associated B-cells is not affected by impaired [in (NOD \times NOR)F1 mice] or accelerated (in 8.3-NOD mice) β -cell death. Moreover in 8.3-F1 mice, the disease hardly progresses to diabetes despite presence of severe, but nondestructive, insulinitis. Again, the islets of these mice appear to contain significantly more nervous system-specific B-cells than islet-specific B-cells. These results lend strong support to the idea that B-cell responses against islet-associated nervous elements may be an early event in diabetogenesis. This observation is consistent with a recent publication indicating that development of type 1 diabetes in both mice and humans is accompanied by T-cell responses against pancreatic nervous system tissue elements (7) and might account for some of the neurological pathologies that have been described in pre-diabetic humans and mice (35–37).

The mechanisms underlying this predominant anti-nervous tissue-specific B-cell response in type 1 diabetes are unclear. β -Cells and neuronal cells share several functional and phenotypical similarities, including the production of a large number of recognized autoantigens, such as GAD and ICA515. Therefore, an initial insult against one of the shared proteins on β -cells may result in an autoimmune attack on the nervous system and vice versa. It has been suggested that during the perinatal period, a defect in physiological nerve phagocytosis by dysfunctional macrophages combined with a temporary β -cell hyperactivity might be determining factors in type 1 diabetes pathogenesis (38). Another possibility is that islet insults induce the expression of nerve growth factors and their receptors in islet cells, leading to reactive gliosis (and immunogenicity) of peri-islet Schwann cells (35). Alternatively, since Schwann cells upregulate both major histocompatibility complex class I and class II molecules in the presence of

proinflammatory cytokines, they might be able to process and present endogenous and exogenous antigens to autoreactive T-cells in situ, rendering them susceptible to T-cell-mediated attack, antigen shedding, and B-cell activation (39,40).

Alternatively, islet-infiltrating B-cells may have tolerogenic, rather than prodiabetogenic, activity on autoreactive T-cells (41–44). This might account for the increased frequency of islet-infiltrating B-cells with islet cell specificity in diabetes-resistant (NOD \times NOR)F1 mice, which might downregulate islet cell-specific T-cell responses (29). This would explain, in part, why monoclonal RAG-2^{-/-} 8.3-F1 mice (which do not export B-cells to the periphery) develop diabetes with a similar incidence and tempo as RAG-2^{-/-} 8.3-NOD mice (30).

Whatever the mechanisms, it is clear that most of the antibody-secreting hybridomas that displayed specificity for pancreatic nervous system elements or islet cells arose from B-cells that had undergone immunoglobulin class switch recombination in vivo. This is in contrast to the overrepresentation of the IgM isotype among antibodies corresponding to tissue staining patterns typically associated with polyreactive natural-like antibodies, such as those specific to exocrine tissue, cell nucleus, or extracellular connective tissue.

These observations provide some clues to the origin of these B-cell responses. In mice, Th1-driven humoral immune responses are characterized by production of antibodies dominated by IgG3 and IgG2a (and probably IgG2c) isotypes. Th2 responses, on the other hand, predominantly give rise to IgG1 and IgE antibodies, whereas Th3 responses typically result in the production of IgG2b and IgA molecules. Our findings point to the coexistence of autoreactive B-cells with a Th1 (IgG2c), Th2 (IgG1), and Th3 (IgG2b) patterns within the islet lymphocyte infiltrate. These results are consistent with the complex cytokine

profiles of islet-associated T-cells in 8.3-NOD, 8.3-NOR, and 8.3-F1 mice (30). In any case, these data indicate the presence of an active B-cell response against pancreatic nervous system antigens at the earliest stages of diabetogenesis (i.e., in diabetes-resistant mice).

We do not yet know whether this intraislet immune response against pancreatic nervous tissue elements also occurs in human type 1 diabetic patients or whether this response is key to diabetogenesis. Nevertheless, since humoral immune responses against autonomous nervous tissue have been described in newly diagnosed type 1 diabetic patients (45–48) and in genetically predisposed individuals (49,50), it is tempting to speculate that our observations are not a peculiarity of NOD mice. Moreover, an early loss of islet sympathetic nerves during the course of the diabetes has been also observed in BioBreeder diabetic rats (26). Nevertheless, cross-reactivity of these murine antibodies for both rat and human pancreas tissue indicates the existence of conserved antigenic determinants across species. Although, at present, we do not know the molecular nature of these antigenic determinants, preliminary data strongly suggest that most monoclonal antibodies representative of each staining pattern recognize native antigenic protein determinants (data not shown).

In conclusion, we describe a set of antibody-secreting hybridomas from islet-infiltrating B-cells of diabetes-prone and -resistant mice. Our results show that most antibody-secreting, islet-infiltrating B-cells recognize antigens expressed by nervous cellular elements of the pancreas and provide evidence for the existence of an active lymphocyte response against these cellular elements early in diabetogenesis. This work lends support to the notion that nervous system elements of islets of Langerhans are important targets of the diabetogenic autoimmune response.

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