Autoantibodies in Nonobese Diabetic Mice Immunoprecipitate 64,000-M_r Islet Antigen

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In insulin-dependent diabetes mellitus (IDDM) in humans and BB rats, islet cell autoimmunities associated with autoantibodies to a β-cell protein of 64,000 M_r (64K) have been described. We report that sera from newly diagnosed nonobese diabetic (NOD) mice similarly contain an autoantibody that immunoprecipitates 64K autoantigen from detergent lysates of [^{35}S]methionine-labeled murine islet cells. The autoantibody was detectable by weaning; it disappeared within weeks after diabetes onset and was absent in older nondiabetic NOD mice as well as all of three non-diabetes-prone control strains tested. The 64K β-cell autoantigen may be a critical target in the immunopathogenesis of IDDM. *Diabetes* 37:1587–90, 1988

The clinical onset of insulin-dependent diabetes mellitus (IDDM) is characterized by inflammation within the pancreatic islets (insulitis) and specific loss of most of the insulin-producing β-cells in the pancreatic islets of Langerhans (1). Most newly diagnosed IDDM patients have islet cell cytoplasmic (ICA)- and/or surface (ICSA)-reactive autoantibodies (2–5); however, the nature of the primary β-cell target autoantigen(s) they react with remains unclear. Patients with diabetes onset during infancy and childhood often have circulating insulin autoantibodies (6,7). Recently, IDDM in humans and BB rats has been characterized by the presence of an autoantibody directed against an immunoprecipitable 64,000-M_r islet cell protein (64K) (8–13). In studies of fluorescence-activated cell-sorted islet cells, Christie et al. (10) have shown 64K to be present predominantly in the β-cell population (10). Furthermore, this protein was not detected in freshly isolated human peripheral lymphocytes or in 11 different endocrine and nonendocrine human cell lines, indicating a cell-restricted expression of the protein (11). The function of this protein in β-cell physiology is not yet known. The 64K autoantibody (64KA) has been observed in ~95% of human IDDM patients at diagnosis, and as early as 8 yr before the clinical onset of IDDM (11,13). Studies have shown that human IDDM has a long prodromal period before the clinical presentation of IDDM (14). This prediabetic period has been difficult to study because many high-risk relatives of affected probands must be screened for circulating autoantibodies to identify the susceptible individuals, and long observation periods are necessary to document the natural history of the β-cell failure in the disease. The nonobese diabetic (NOD) mouse is a useful animal model for human IDDM that may provide important insights into the sequence of pathogenic events and lead to an understanding of the nature of the target islet cell autoantigens involved in the autoimmunological process. We therefore studied NOD mice for possible 64KAs both before and after IDDM onset.

MATERIALS AND METHODS

Sera were obtained from both male and female NOD and control (BALB/c, C57BL/6, C3H/HeJ) strains of mice of various ages. Diagnosis of IDDM was characterized by thirst, weight loss, and persistent hyperglycemia of >240 mg/dl. All received daily insulin doses of protamine zinc insulin (Lilly, Indianapolis, IN) at 0.5–2.0 U/day. All mice were obtained from the Department of Pathology mouse colony (Univ. of Florida College of Medicine, Gainesville, FL).

BALB/c islets were isolated according to the method of Brunstedt et al. (15), with yields of ~50 islets per adult mouse, and labeled with [^{35}S]methionine according to the method of Baekkeskov et al. (9). Islet cells (500 islets/sera sample) were washed twice (4°C) in supplemented RPMI-1640 medium (Gibco, Grand Island, NY; 2.0% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin) followed by one wash in buffer containing 20 mM Tris (pH 7.4),

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150 mM NaCl, 1000 KIE/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride. Cells were then lysed in the buffer containing 1% Nonidet P-40 (NP40; vol/vol). Insoluble material was removed by ultracentrifugation (100,000 × *g* for 30 min). The lysate was incubated with normal mouse serum (10 μl/100 μl supernatant for 1 h at 4°C) followed by adsorption to an excess of protein A–Sepharose CL-4B (Pharmacia, Piscataway, NJ). This incubation-adsorption procedure was repeated three times. Aliquots (100 μl) of unbound (precleared) lysate were incubated with 25 μl of either NOD or control sera for 18 h (4°C). To each assay tube, 100 μl of preswollen protein A–Sepharose CL-4B was added, and the reaction mixture was incubated for 45 min (4°C). The protein A–Sepharose CL-4B was then washed five times by centrifugation (200 × *g*, for 10 s) in 0.5% NP40 buffer and once in cold water (200 × *g* for 30 s). Bound immune complexes were denatured by boiling for 5 min in sample buffer containing 80 mM Tris (pH 6.8), 3.0% (wt/vol) sodium dodecyl sulfate (SDS), 15% sucrose, 0.001% (vol/vol) bromphenyl blue, and 5.0% (vol/vol) β-mercaptoethanol. The Sepharose beads were removed by centrifugation (200 × *g* for 1 min), and the supernatant was electrophoresed through 12%–SDS polyacrylamide separating gels followed by Coomassie blue staining and autoradiography.

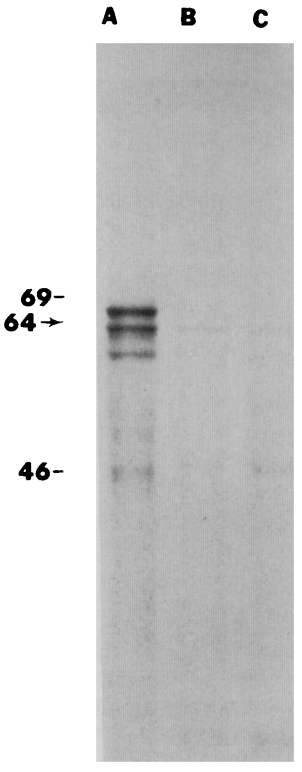

FIG. 1. Autoradiograph of sodium dodecyl sulfate–polyacrylamide gel electrophoresis of immunoprecipitates of murine islet cell extracts with newly diagnosed diabetic nonobese diabetic (NOD) and control sera. Each *band* represents protein bound by either immunoglobulin or protein A–Sepharose CL-4B. Molecular-weight markers are indicated at *left*. Antigen of 64,000 *M*, indicated by *arrow*. *Lane A*, 64,000-*M*, protein recognized by sera from newly diagnosed NOD mouse. *Lanes B and C*, control sera failed to immunoprecipitate 64,000-*M*, protein from islet cell extract.

RESULTS

The initial studies were to learn whether 64KA occurred in NOD mice. Sera were obtained from 8 female NOD mice with newly diagnosed IDDM (mean age at onset 104.5 ± 13.5 days, range 93–134 days) and 11 healthy control mice (2 males, 9 females, mean age 97.7 ± 30.4 days, range 50–120 days) of BALB/c (n = 5), C57BL/6 (n = 4), and C3H/HeJ (n = 2) strains. Table 1 shows that 64KA was detected in 87% of NOD at diabetes onset, but it did not occur in any of the non-diabetes-prone control strains (P <.001). Figure 1 (*lane A*) shows the 64K protein (*arrow*) recognized by NOD sera. In contrast, control sera in Fig. 1, *lanes B* and *C*, failed to immunoprecipitate 64K protein from the islet cell extract. Bands other than 64KA primarily represent cellular components that were nonspecifically absorbed to protein A–Sepharose or immunoglobulin and were uniformly present in both NOD and control lanes. The major component bands of 55KA and 45KA may represent tubulin and actin, respectively (8).

To learn about the natural history of 64KA with respect to IDDM, serum samples obtained from five mice at weaning (2 male, 3 female, aged 23–25 days) were analyzed, and their pancreases were examined histologically for insulitis.

TABLE 1
Frequency of insulitis and 64,000-*M*, protein autoantibodies within 3 study groups

Group	*n*	Insulitis	Positive 64,000-*M*, autoantibodies (%)
Control	11		0 (0)
Weaning NOD	5	3/4	4 (80)*
Newly diabetic NOD	8	3/3	7 (87)†

Controls were non-diabetes-prone BALB/c, C57BL/6, and C3H/HeJ mice. NOD, nonobese diabetic.
*P < 0.01, †P < 0.001, significance by Fisher's exact analysis of indicated groups versus controls.

Insulitis was defined as an unequivocal intraislet infiltrate of lymphocytes on hematoxylin-and-eosin–stained pancreatic sections containing five or more islets. The 64KAs were already present in 80% (4 of 5) of the mice. Concomitantly, early insulitis was also present in three of the four mice whose pancreatic histologies were analyzed. In contrast, two animals (both male) studied at ≥190 days of age who had never developed IDDM had no 64KA, albeit both had insulitis. An autoradiograph of the 64K protein immunoprecipitated by a serum sample from a 24-day-old male NOD mouse and that of serum similarly assayed from a 190-day-old male NOD mouse that lacked 64KA and failed to develop spontaneous IDDM is displayed in Fig. 2. To learn how long the 64KA persisted beyond disease onset, sera from nine diabetic NOD mice (2 male, 7 female; mean age at onset 103.2 ± 18.1 days, range 80–124 days; mean duration of diabetes 26.9 ± 10.0 days, range 19–40 days) were analyzed, and only two of the nine samples taken from mice 19 days after onset were positive.

DISCUSSION

In our colony, clinical symptoms of diabetes are detectable in females beginning by ~12 wk of age; by 30 wk of age, ~80% of females and 50% of males are overtly diabetic. The timing of insulitis and IDDM in NOD mice has been well described (16–18). There is an infiltration of monocytic cells in both males and females at 2–4 wk of age that signals the

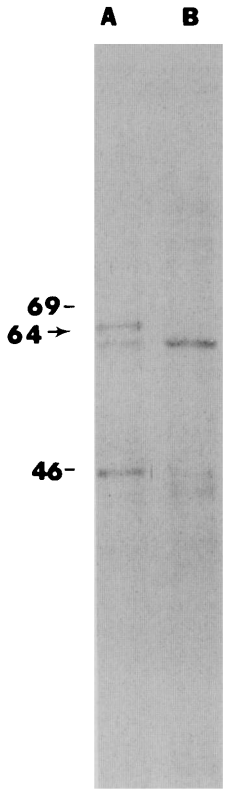

FIG. 2. Autoradiograph of sodium dodecyl sulfate–polyacrylamide gel electrophoresis of immunoprecipitates of murine islet cell extracts with sera from 24-day-old (*lane A*) and 190-day-old (*lane B*) nondiabetic nonobese diabetic mice. Only diabetic mouse sera immunoprecipitated 64,000-M_r protein.

onset of islet destruction and insulin loss. The earliest infiltrates are seen in the margins of islets located near blood vessels. Kanazawa et al. (19) report these infiltrates to be primarily composed of IgM^+ B-lymphocytes, organized in follicle-like structures, with fewer T-lymphocytes of both the $CD4^+$ (helper) and $CD8^+$ (suppressor/cytotoxic) phenotypes and virtually no natural killer (asialo-G_{M1}^+) cells. Miyazaki et al. (20) also found extensive B-lymphocyte infiltration of NOD islets that increased with progression of active disease. At the very early stages of insulitis, the latter group also reported a higher proportion of $CD4^+$ T-lymphocytes of which as many as half were class II MHC positive, indicating their state of activation. Whereas the islet cell destruction is progressive and detectable with selective loss of β-cells, the antigen(s) responsible for this early immunological aberration has not been identified.

In this study, ~87% of newly diabetic NOD mice were observed to possess 64KA. The presence of 64KA in a high percentage of NOD mice with newly diagnosed IDDM in conjunction with the absence of 64KA in age-matched nondiabetes-prone control mice suggests that 64KA is specific for IDDM and its prodromal state. The presence of 64KA was also one of the events detected initially, present even at weaning.

We have previously reported that NOD mice contain antibodies against bovine serum albumin (BSA; 21). Evidence to separate these BSA antibodies from the 64KA we observed is fourfold. First, BSA is used as a molecular-weight marker (Amersham, Arlington Heights, IL) and clearly has a higher mobility (69,000 M_r) than 64KA. Second, our studies only utilize metabolically labeled proteins, and any BSA present would not label (or would be separated by our β-mercaptoethanol treatment) in such a procedure. Third, the time course of appearance of BSA antibodies is later than 64KA in that the latter occur at the time of weaning, before consumption of laboratory chow containing BSA. Finally, Colman et al. (12) have shown that the 64K protein is clearly separate from BSA, and the recovery of 64K protein by use of diabetic sera was unaffected by preclearing extracts with anti-BSA antibodies.

The pathogenic potential of an islet cell surface–expressed 64K antigen is great. If an antigen-driven autoimmune reaction is responsible for the specific destruction of pancreatic β-cells, the eliciting autoantigen would seem to be specifically expressed at the surface of β-cells. However, Colman et al. (12), with methods that were successful in surface labeling insulin and insulin-like growth factor I receptors on RINm5F cells, were unsuccessful in surface labeling 64K autoantigen on human islet cells, thereby questioning the surface expression of 64K autoantigen. Baekkeskov et al. (11) have, however, argued that because the 64K autoantigen is only observed in detergent-phase (hydrophobic) extracts of islet cells and can coprecipitate with other known surface molecules (S. Baekkeskov, unpublished observation), the 64K autoantigen represents a surface-expressed molecule. Using Triton X-114 human islet preparations, we also only observe 64K autoantigen within the detergent phase and not during the aqueous (hydrophilic) phase (data not shown). With mouse islet preparations, we plan experiments with lactoperoxidase surface labeling to study whether the 64K autoantigen we observed is expressed on the surface of islet β-cells.

Furthermore, if such an antigen is the target of a primary specific immune response to β-cells, the antibodies should be detectable at the initial phase of the insulitis lesion and precede secondary immune phenomena possibly including ICA. In humans, ICA and ICSA tend to disappear with the progression of the disease when complete elimination of the pancreatic autoantigen would be expected (22). In our studies, autoimmune 64KA reactivity declined rapidly after onset of diabetes, suggesting that elimination of islet cells expressing the 64,000-M_r protein had occurred. The data taken together suggest that the 64K autoantigen may be primarily involved in the β-cell autoimmunity of the NOD mouse.

In IDDM seen in BB rats and humans, 64KA similarly appears early in the natural history of the disease and shows considerable specificity as a marker for IDDM. The finding of 64KA in all three species with spontaneous IDDM argues that autoimmunity directed to this autoantigen may be of considerable pathogenic importance and that the 64,000-M_r protein may have a vital physiological function in normal β-cells.

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