Predictive Value of Lymphocyte Antibodies for the Appearance of Diabetes in BB Rats

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Lymphocyte antibodies have been described in autoimmune disorders, including insulin-dependent diabetes mellitus (IDDM). We have developed a quantitative method to measure autoantibodies directed against T-lymphocytes, based on two-color fluorescence labeling of Wistar mononuclear cells and analysis of fluorescence by flow cytometry. The lymphocyte antibody levels were determined retrospectively in the serum of 73 BB and 18 Wistar rats. We demonstrated the binding of the lymphocyte autoantibodies of both $CD4^+$ and $CD8^+$ T-cells. Lymphocyte antibodies were present in 90% of the BB rats at diabetes onset, compared with 11% of the Wistar rats. At 75 days of age, 83% of the BB rats, which later became diabetic, were positive for the lymphocyte antibodies, compared with 15% of their littermates who maintained a normal glucose tolerance. In all cases, the antibodies were of the immunoglobulin M isotype. We conclude that lymphocyte antibodies are present before diabetes onset and, using this method, that their presence can predict the development of diabetes with a sensitivity of 83% and a specificity of 85% in BB rats. *Diabetes* 43:137–42, 1994

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IDDM, insulin-dependent diabetes mellitus; Ig, immunoglobulin; IgM, immunoglobulin M; IgG, immunoglobulin G; ICSA, islet cell surface antibodies; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; OX19/PE, PE-conjugated OX19; MoAb, monoclonal antibody; FACS, fluorescence-activated cell sorter; CV, coefficient of variation.

Lymphocyte antibodies have been described in several idiopathic diseases (1). In the BB rat, they are present before insulitis and diabetes onset along with circulating islet cell surface antibodies (ICSA) (2,3). They are considered to be a manifestation of altered humoral immunity in insulin-dependent diabetes mellitus (IDDM) (2,3). The stimulus for their production is still unknown, but their increased prevalence in relatives of patients with IDDM (4) or other autoimmune diseases and in patients with acute viral infection suggest that it may be related to the environment (1). The role of lymphocyte antibodies in the pathogenesis of diabetes and the specific cell subset targeted by these antibodies in the BB rat have not been defined as yet. Studies that used splenocytes and radioligand (2,3,5) or cytotoxicity (1,4,6) assays revealed a high prevalence of lymphocyte antibodies in animals and in humans developing diabetes, compared with their normal control subjects. They also demonstrated a positive correlation between lymphocyte antibodies and ICSA. However, the predictive value of lymphocyte antibodies for the development of diabetes among predisposed subjects has never been demonstrated.

We developed a new method based on two-color fluorescence labelling of mononuclear cells and two-color flow cytometry. In this study, we report the predictive value of the detection of lymphocyte antibodies by this new method for the development of diabetes in the BB rat.

RESEARCH DESIGN AND METHODS

BB rats were obtained from Dr. P. Thibert (Animal Resources Division, Health and Welfare Canada, Ottawa). Wistar rats were purchased from Charles River Laboratories of Canada (St-Constant, Quebec). The rats were kept in metabolic cages in laminar flow hoods and were

housed in rooms controlled for humidity (70%) and temperature (20°C), with 12-h light-dark cycles. They were fed ad libitum (rat chow, Ralston-Purina, St-Louis, MO).

Blood was sampled by cardiac puncture under light ether anesthesia from recently diagnosed diabetic BB rats (within 5 days of glycosuria onset) (n = 20), Wistar rats (n = 18), and 150-day-old diabetic (n = 14) and euglycemic (n = 14) littermates for lymphocyte antibody analysis. The sera were separated and kept frozen. Frozen samples from 75-day-old diabetes-prone BB rats (n = 25), which were followed until 150 days of age as controls in an intervention study, were also used. The sera were then thawed, centrifuged (15 min at 1800 g), and used for lymphocyte antibody measurements. The lymphocyte antibody measurements were performed with peripheral blood mononuclear cells from other Wistar rats used as targets. Blood was sampled by cardiac puncture under ether anesthesia, diluted 1:3 with phosphate-buffered saline (PBS) with fetal calf serum 5%, and layered as 3 ml aliquots on 3 ml of Ficoll-Hypaque (165 ml of 9 g/dl Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden) with 69 ml of 34.5 g/dl Sodium Hypaque (Winthrop Laboratories, Aurora, Ontario) density = 1.077 g/ml). After centrifugation at 400 g for 30 min, cells were extracted and washed twice with PBS (100 g for 10 min). The final pellet of cells was resuspended in 0.5 ml of PBS, and cell concentration was measured with a hemocytometer. The cells (3.5×10^5) were incubated (30 min at 4°C) with 60 μl of undiluted serum. After 3 washes (100 g), the cells were incubated (30 min at 4°C) with 50 μl of fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat immunoglobulin G (IgG) and immunoglobulin M (IgM) antibody (Bio/Can Scientific, Mississauga, Ontario) at a dilution of 1:40 to determine cell-bound immunoglobulin (Ig). After 3 other washes (100 g), they were incubated (20 min at 4°C) with 5 μl of PE-conjugated OX19 (OX19/PE) (Serotec, Bicester, England) (dilution 1:50, $CD5^+$ T-cells).

To determine the target T-lymphocyte subsets against which the lymphocyte antibodies were directed, we repeated the experiment using either 5 μl of OX19/PE and 20 μl of FITC-conjugated W3/25 (W3/25-FITC) (Serotec) (dilution 1:20, $CD4^+$ T-cells and macrophages/monocytes) or 5 μl of OX19/PE and 20 μl of FITC-conjugated OX8 (OX8-FITC) (Serotec) (dilution 1:20, $CD8^+$ T-cells, macrophages/monocytes, natural killer cells) during the third incubation. To determine whether the anti-lymphocyte antibody isotype was IgG or IgM, we measured the antibodies in the second incubation, using FITC-conjugated mouse anti-rat IgG, Fc-fragment-specific antibody (Jackson Immuno Research Laboratories, West Grove, PA) or FITC-conjugated mouse anti-rat IgM Heavy Chain (MARM-4 FITC) (Serotec).

The cell fluorescence readings were performed with a fluorescence-activated cell sorter (FACS) Scan Analyzer (Becton Dickinson, Sunnyvale, CA), using an air-cooled 15 mW argon ion laser at 488 nm and analyzed by a Consort 30 computer system (Becton Dickinson). Before reading, propidium iodide was added to the samples (10 μg/ml), and the propidium iodide-positive cells (dead cells) were gated out at acquisition. The acquisition was performed on the lymphocyte area of the scatterplot using a gate. In each set of experiments, the serum was replaced by 60 μl of PBS in the first incubation to set the markers. The marker in red fluorescence was set to allow the best separation of $CD5^+$ ($OX19^+$) and $CD5^-$ ($OX19^-$) cells. The marker in green fluorescence was set to confine the target population to quadrant 1; channel 350 for CD4 T-cells ($CD8^-$ T-cells, OX8FITC/OX19PE); channel 350 for CD8 T-cells ($CD4^-$ T-cells, W3/25FITC/OX19PE); and channel 220 for total T-cells (OX19PE). These markers were kept for all serum measurements.

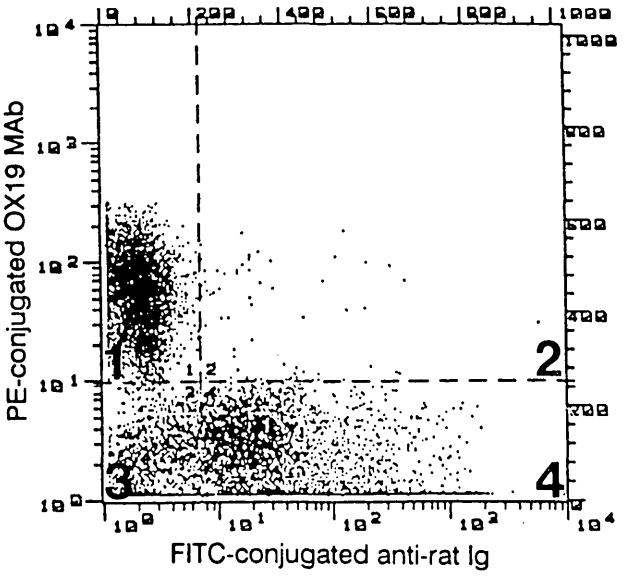

FIG. 1. FACS Scan analysis of OX19-PE and anti-rat Ig-FITC binding to Wistar mononuclear cells in the absence of serum. The cells are plotted according to the intensity of their fluorescence with OX19-PE on the vertical axis and FITC-conjugated anti-rat Ig on the horizontal axis. The T-lymphocytes, found in quadrant 1, are negative for the anti-rat Ig because they have no Ig at their surface.

Statistical analysis. The data were analyzed using unpaired Student's t tests when two groups were compared. Sensitivity and specificity of the tests were calculated using the young, diabetes-prone BB rat data. Results were considered significant for $P < 0.05$.

RESULTS

Figure 1 shows the analysis of FITC-conjugated anti-rat Ig and OX19/PE with Wistar mononuclear cells without previous incubation with serum. The dots represent the fluorescent cells plotted according to the intensity of their fluorescence, with OX19/PE on the vertical axis and FITC-conjugated anti-rat Ig on the horizontal axis. Quadrant 1 contains all T-lymphocytes ($OX19^+$ + anti-rat Ig^-), revealing that no rat antibody is bound at the surface of these T-lymphocytes. Quadrants 3 and 4 contain the monocytes, natural killer cells, and B-lymphocytes ($OX19^-$). To determine the presence of lymphocyte antibodies, we looked at the variations in T-lymphocyte fluorescence with the anti-rat Ig after incubation with different sera. In the presence of lymphocyte antibodies, the T-lymphocytes shifted into quadrant 2 (became positive with the FITC-conjugated anti-rat Ig) after being

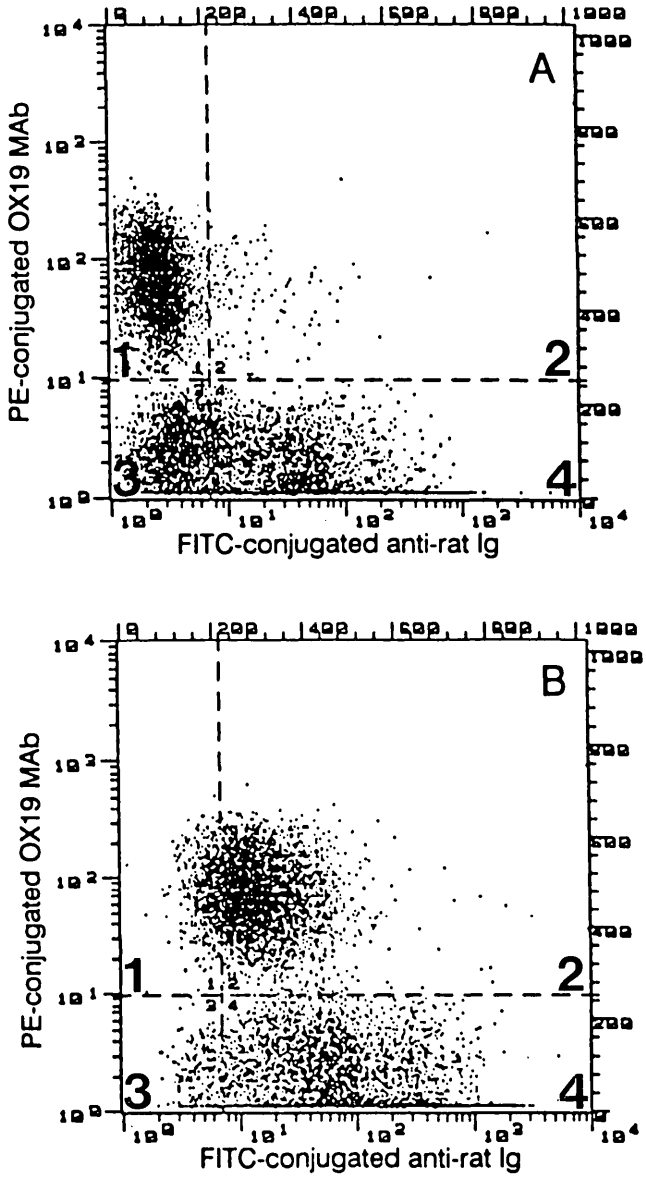

FIG. 2. FACS Scan analysis of OX19-PE and anti-rat Ig-FITC binding to Wistar mononuclear cells after incubation with negative serum from a Wistar rat (*A*) and with positive serum from a diabetic BB rat (*B*). With the positive serum, the initially anti-rat Ig$^-$ T-cells become anti-rat Ig$^+$ (quadrant 2) after incubation.

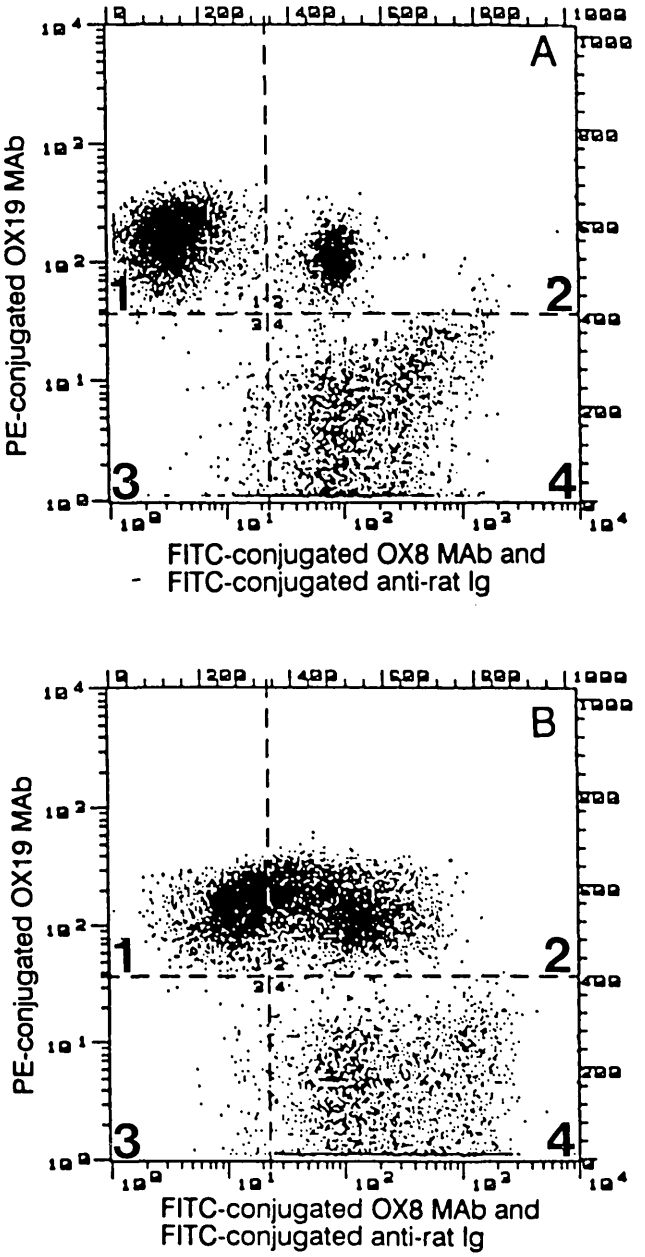

FIG. 3. Binding of antilymphocyte antibodies on CD4$^+$ T-cells. The CD4$^+$ T-cells (CD8$^-$ T-cells) were isolated in quadrant 1 by the addition of OX8-FITC and OX19-PE. These cells that were negative with Wistar serum and FITC-conjugated anti-rat Ig (quadrant 1) (*A*) became positive with diabetic BB rat serum and anti-rat Ig (quadrant 2) (*B*).

incubated with that serum (Fig. 2). To assess whether the anti-lymphocyte antibodies could bind to CD4$^+$ T-cells (CD8$^-$ T-cells), we isolated these cells in quadrant 1 using the combination of OX8-FITC and OX19/PE. Although these cells remained fluorescein negative (quadrant 1) with Wistar serum and FITC-conjugated anti-rat Ig (Fig. 3*A*), the majority of CD4$^+$ T-cells became fluorescent (quadrant 2) with the addition of serum from a diabetic BB rat and FITC-conjugated anti-rat Ig (Fig. 3*B*).

Similarly, the CD8$^+$ T-cells were isolated in quadrant 1 by the combination of W3/25-FITC and OX19-PE. A majority of CD8$^+$-T cells (CD4$^-$ T-cells), which were fluorescein negative (quadrant 1) with Wistar serum and FITC-conjugated anti-rat Ig (Fig. 4*A*), became fluorescent (quadrant 2) after the incubation with the serum from a diabetic BB rat and the FITC-conjugated anti-rat Ig (Fig. 4*B*).

The latter method, using CD8$^+$ T-cells as targets, was applied to a series of samples from 6 different groups of rats to determine their level of lymphocyte antibodies. This level was defined as the percentage of CD8$^+$ T-cells that became fluorescent with each sample. Figure 5 shows the data for the 6 groups. A total of 71.4 ± 5.7% of the CD8$^+$ T-lymphocytes became fluorescent with the FITC-conjugated anti-rat Ig after their incubation with the sera from the diabetic animals, compared with

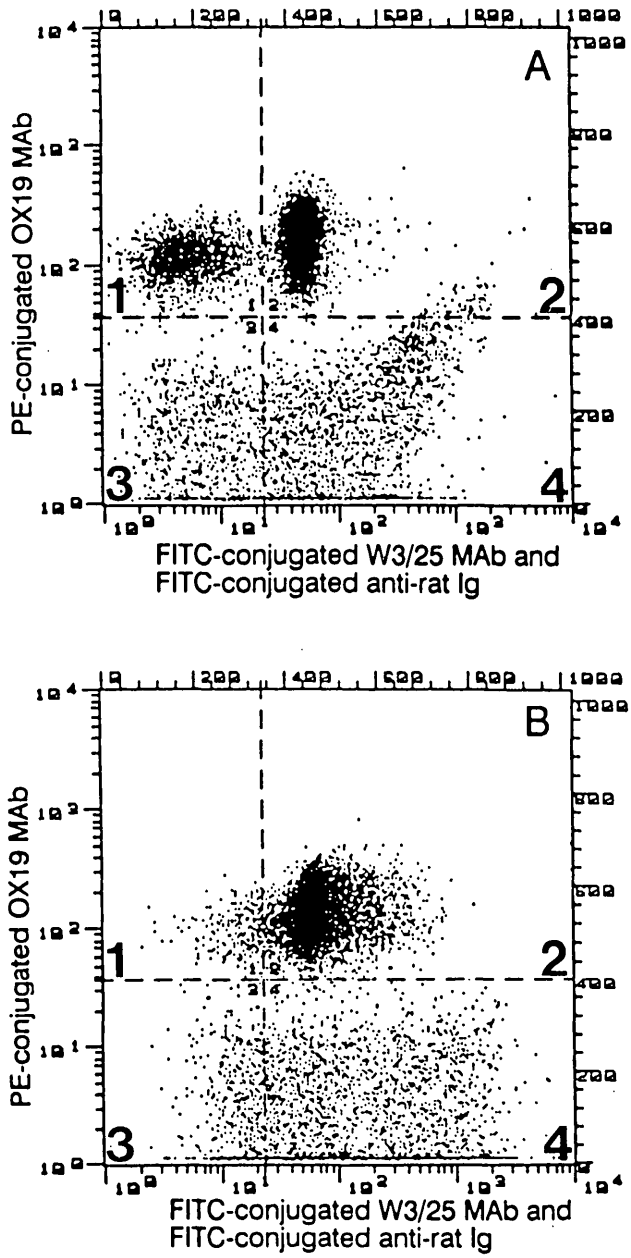

FIG. 4. Binding of antilymphocyte antibodies on CD8$^+$ T-cells. The CD8$^+$ T-cells (CD4$^-$ T-cells) were isolated in quadrant 1 by the addition of W3/25-FITC and OX19-PE. These cells that were negative with Wistar serum and FITC-conjugated anti-rat Ig (quadrant 1) (*A*) became positive with a diabetic rat BB serum and anti-rat Ig (quadrant 2) (*B*).

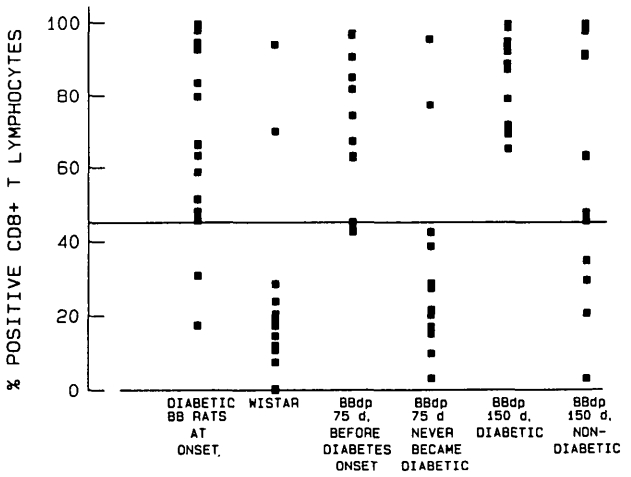

FIG. 5. Lymphocyte antibody binding to CD8$^+$ T-lymphocytes after the incubation of mononuclear cells from Wistar rats with sera from 6 different groups of rats and FITC-conjugated anti-rat Ig. The sera were considered positive for the presence of lymphocyte antibodies if >45% of the initially negative CD8$^+$ T-cells became fluorescent for anti-rat Ig after being incubated with these sera.

20.1 ± 5.9% after the incubation with the sera from the Wistar rats ($P < 0.001$).

Among the 25 diabetes-prone BB rats tested at 75 days of age, 12 developed diabetes and 13 maintained a normal glucose tolerance. The sera from the rats that eventually developed diabetes resulted in 70.7 ± 5.8% of the target mononuclear cells becoming fluorescein positive, compared with 32.1 ± 7.4% ($P < 0.001$) for the sera from the rats that remained normoglycemic.

To define lymphocyte antibody positivity, a cutoff level of 45% of target mononuclear cells achieving fluorescent was chosen, because this allowed the best separation of rats that eventually developed diabetes from those that did not. The intra-assay coefficient of variation (CV) was 1.9% for the positive serum samples and 5.1% for the negative serum samples. The interassay CV was 10.1% for the positive serum samples ($n = 9$) and 15.4% for the negative serum samples ($n = 9$).

Of the 20 BB rats tested at onset of diabetes, 18 (90%) were positive for lymphocyte antibodies, compared with 2 (11%) of the 18 Wistar rats studied. Elevated antibody levels were found in 10 (83%) of the 12 diabetes-prone BB rats that later developed diabetes, compared with 2 (15%) of their 13 littermates who maintained normal glucose tolerance. In this group, diabetes could be predicted with a sensitivity of 83% and a specificity of 85%. At 150 days of age, all 14 diabetic rats and 10 (71%) of the 14 nondiabetic diabetes-prone BB rats had high lymphocyte antibody levels (86 ± 3.3 vs. 63.2 ± 8.9%).

We determined the isotype (IgG versus IgM) of lymphocyte antibodies using specific anti-IgG or anti-IgM fluorescent antibodies in the third incubation. None of the samples examined was positive for IgG antibodies. An excellent correlation was found between the lymphocyte antibody levels and the IgM lymphocyte antibody levels ($r = 0.746$, $P < 0.001$, $n = 46$). All sera positive for Ig were also positive for IgM lymphocyte antibodies.

DISCUSSION

We report, for the first time, a predictive value of lymphocyte antibodies for the development of diabetes in the BB rat. Using the serum from 75-day-old diabetes-prone BB rats, we were able to predict the development of diabetes with a sensitivity of 83% and a specificity of 85%.

Our lymphocyte antibody assay involves the incubation of peripheral blood mononuclear cells (from Wistar rats, as targets) with the sera to be tested. A fluorescent labeling of the surface-bound Ig is then performed with an FITC-conjugated anti-rat Ig. OX19/PE monoclonal

antibody (MoAb), which is specific for the T-lymphocytes, was used to isolate T-cells from other mononuclear cells to avoid any confusion between the expected binding of the anti-rat Ig with the B-lymphocytes and monocytes and the binding of the anti-rat Ig with the autoantibodies on the target T-lymphocytes. The use of a fluorescent cell analyzer allowed us to read faint fluorescence in an objective and quantitative manner.

Our results confirm previous studies (2,3,5) using Wistar rat splenocytes as targets in radioligand assays that reported high levels of lymphocyte antibodies in diabetes-prone BB rats before and at diabetes onset. This permitted us to distinguish them from age-matched Wistar rats and BB rats with an expected low incidence of diabetes. These studies also described a positive correlation between the lymphocyte antibodies and the ICSA. They were both associated with insulitis, but could not predict the development of diabetes. The sensitivity of the FACS Scan reading of the fluorescence and the discrimination of the target T-lymphocytes may explain our ability to predict diabetes in the young, diabetes-prone BB rats.

However, it was impossible to distinguish between the diabetic and the nondiabetic diabetes-prone BB rats by testing their sera at 150 days of age. Lymphocyte antibody levels were elevated in both groups. This could be related to an increase of the antibody levels with age in the nondiabetic, diabetes-prone BB rats, reflecting an ongoing but less severe autoimmune process associated with insulitis in the absence of overt diabetes. It might also represent a normal phenomenon with aging in rats.

In this study, we describe the binding of lymphocyte antibodies to both $CD4^+$ and $CD8^+$ T cells. This has also been described in humans with systemic lupus erythematosus (7). Furthermore, in these patients, the lymphocyte antibodies were reported to cause a depletion in the T-cell subset to which they were found to be directed. The diabetes-prone BB rat has a severe T-lymphopenia present from birth that persists throughout life and involves both subsets (8–10). It has already been suggested that lymphocyte antibodies could be related to this lymphopenia (2). Our study does not directly address the question of the role of the lymphocyte antibodies in the pathophysiology of the BB-rat lymphopenia. However, the lymphopenia is present in these rats independently of their metabolic outcome. The predictive value of lymphocyte antibodies that we found in 75-day-old BB rats suggests that they are not responsible for the severe T-lymphopenia of the BB rat.

The lymphocyte surface antibodies belong to the IgM class. We have not demonstrated IgG lymphocyte antibodies in this study. In humans, the isotype has been shown to be primarily IgM, with 27% of IDDM patients having IgM lymphocyte antibodies, whereas 7% had IgG lymphocyte antibodies (11).

The significance of these autoantibodies remains unknown. They have been described mainly in diseases of viral (12) or autoimmune origin, possibly implicating a genetic predisposition, such as IDDM (13), systemic lupus erythematosus (14), multiple sclerosis (15), rheumatoid arthritis (16), Hodgkin's disease (17), or inflammatory bowel disease (18). They have also been reported in relatives of IDDM patients (4), systemic lupus erythematosus (19), and inflammatory bowel disease (18), irrespective of consanguinity. This suggests that lymphocyte antibodies might be induced by an environmental agent, such as a virus that may also induce the pathological immune process in genetically predisposed subjects. They may also be a marker of the activation of the whole autoimmune process in these individuals.

Independently of their significance or pathogenic role, we demonstrated a predictive value of the presence of autoantibodies binding to T-lymphocytes for the appearance of diabetes in BB rat littermates.

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