TWO GENES REQUIRED FOR DIABETES IN BB RATS

Evidence from Cyclical Intercrosses and Backcrosses

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The BB rat strain spontaneously develops diabetes mellitus which rapidly progresses to ketoacidosis and death if not treated with exogenous insulin. 40–60% of BB rats develop overt diabetes and exhibit prominent insulitis, selective pancreatic beta cell destruction, and circulating antibodies to islet cell surfaces (1, 2). Diabetes can be prevented with multiple forms of immunotherapy and can be transferred with activated splenic lymphocytes (3, 4). In addition to the diabetic syndrome, BB rats have a striking lymphophenia characterized by an almost total lack of circulating T cells (5). Colle et al. (6–8), using a sequential breeding program, have described associations between the development of diabetes and the inheritance of major histocompatibility complex (MHC)[1] genes, lymphopenia, and susceptibility to pancreatic lymphocytic infiltrates.

One of us (R. M. W.) initiated a breeding program, different from that of Dr. Colle and co-workers, to isolate the BB rat's diabetogenic genes on the genetic background of three inbred rat strains. BB rats were cyclically backcrossed-intercrossed then backcrossed with Brown Norway (BN), Lewis (L), and Wistar-Furth (WF) rats while selecting for diabetes. We studied the expression of lymphopenia and MHC antigens. We will present evidence that at least two independent genes or gene complexes are absolutely necessary for the inheritance of diabetes in these rats. One gene (*l*), determines the T cell lymphopenia of the BB rat and is not linked to the rat MHC. The second gene (RTI-DM), determines the susceptibility to diabetes and is closely linked to the rat MHC.

Materials and Methods

Breeding Program. Outbred diabetic BB rats and the animals used in this study were maintained at the University of Massachusetts Medical Center, Worcester, MA. All animals

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[1] *Abbreviations used in this paper:* BC, backcross; 1BC, first backcross generation; 2BC, second backcross generation; BN, Brown Norway; IC, intercross; 1IC, first intercross generation; L, Lewis; MHC, major histocompatibility complex; WF, Wistar-Furth.

were given *ad libitum* rat chow and water, and were observed for the occurrence of diabetes by twice weekly Testape (Lilly, Indianapolis, IN) urine determination. Only animals that lived to 150 d were included in the statistics for this study. After 150 d animals were no longer tested for diabetes, and were usually sacrificed if not needed for breeding. Diabetic animals were treated with once daily subcutaneous injections of insulin, and this dose was adjusted in relation to Testape findings. Diabetic BB/W rats were bred with inbred strains (BN, L, and WF) producing F1 animals. These were intercrossed to produce an F2 generation. Diabetics in the F2 generation were mated with an animal of the original inbred strain producing the first backcross generation (1BC). These animals were interbred to produce the first intercross generation (1IC). Diabetic intercrosses were mated with rats from the original inbred strain producing the second backcross generation (2BC) and this cyclical process repeated, selecting for diabetes among the intercross animals. No diabetes was observed in F1 or backcross generations.

Mononuclear Cell Isolation. Rats were bled (0.5–1.0 ml) from the tail into heparinized Natelson tubes. Blood was diluted to 5 ml with normal saline and underlaid with 2 ml Ficoll-Hypaque (24:10 vol/vol, 14% Ficoll: 34% Hypaque M; sp gr 1.083). Lymphocytes and monocytes present at the gradient interface were harvested and washed once with a 10-fold excess of RPMI plus G (RPMI 1640 media plus 4 μg/ml gentamycin sulfate). Cells were aliquoted in 30-μl volumes into 12 × 75 mm glass tubes for lymphocyte subset determination and RT1 typing.

Lymphocyte Subsets. Lymphocyte subsets were determined by indirect immunofluorescence. Mouse monoclonal antibody ascites W3/25, W3/13, OX8, and OX6 were obtained from Accurate Chemical (Westbury, NY); OX19 was the kind of gift of Dr. Alan Williams (Oxford, England); P3X63 ascites was produced in BALB/c mice in our own laboratory and used as a negative control. Ascites was diluted 1:100 in RPMI plus G plus 0.5% bovine serum albumin (BSA). Mononuclear cells were incubated with 50 μl of diluted monoclonal antibody for 45 min at 4°C and then washed twice with 1 ml RPMI plus G. A second incubation was done with 50 μl fluorescein-conjugated $F(ab')_2$ goat anti-mouse IgG (Tago Inc., Burlingame, CA) diluted 1:10 in rat serum for 45 min at 4°C. Cells were again washed twice with RPMI plus G. Lymphocyte population percentages were determined using a flow cytometer as described below or with a Leitz-Diavert microscope with fluorescein filters.

RT1 Typing. The expression of class I MHC antigens (encoded by the RT1.A locus in rats) was determined by indirect immunofluorescence. Rat monoclonal antibodies reacting with $RT1^u$ (expressed by BB and WF rats), $RT1^l$ (L), and $RT1^n$ (BN) (LW4.13, 4F12.3, and P4G8, respectively) were developed in Dr. Carpenter's laboratory. Pooled supernatants were diluted 1:10 in RPMI plus G plus 0.5% BSA. Mononuclear cells were incubated with 30 μl diluted supernatant or 30 μl of 1:100 P3X63 ascites for 45 min at 4°C and then washed twice with RPMI plus G. A second 45-min incubation at 4°C was done with fluorescein-conjugated rabbit anti-rat IgG (Cappel Laboratories, Cochranville, PA) diluted 1:20 in RPMI plus G plus 0.5% BSA. The cells were again washed twice with RPMI plus G. RT1 antigen expression was determined by internal comparisons of the patterns generated by flow cytometry with the four monoclonal antibodies described in this section.

Flow Cytometry. A Coulter Epics V flow cytometer (Coulter Electronics, Hialeah, FL) was used in all fluorescence studies. A single beam argon laser (488 nm) was operated at 400 mW and focused with a confocal lens. Forward angle light scatter and log integral green fluorescence were simultaneously recorded. The instrument was aligned and calibrated using fluorescein-conjugated latex beads (Coulter Electronics). A 515-nm long-pass blocking filter, 560-nm dichroic mirror, and a 560-nm short-pass filter system were used in fluorescence determinations. The light scatter amp gain was set at 5, and the high voltage adjustment for the fluorescent photomultiplier was set at 650. After gating on forward angle light scatter, $2.5–5 \times 10^3$ cells were analyzed per run. Similar results were obtained using a Leitz-Diavert microscope with fluorescein filters.

Results

Table I shows the occurrence of diabetes in the breeding program. No F1 or backcross (BC) animals developed diabetes (0/712). Overall 1.7% (71/4253) of F2 and intercross (IC) animals developed diabetes by 150 d of age. There was no significant variation in the incidence of diabetes among strains. Likewise there was no statistically significant variation in the incidence of diabetes among intercross generations.

We studied 99 animals from 17 IC litters for the expression of lymphopenia. We considered rats to be lymphopenic if fewer than 8% of their circulating mononuclear cells were W3/25+. Normal rats have > 25% W3/25+ circulating cells. W3/25+ cells are important in graft-vs.-host disease and mediate help in antibody responses to haptens (9). The IC rats segregated into two nonoverlapping groups with respect to the expression of W3/25 on mononuclear cells (Fig. 1). 24% (24/99) of IC animals were severely lymphopenic. There was no significant strain variation, as 26% (11/42) of Wistar-Furth, 26% (8/31) of Brown Norwegian, and 19% (5/26) of Lewis IC animals were lymphopenic by our criteria. No BC animals (0/35) had decreased percentages of W3/25+ mononuclear cells. The 24% overall prevalence of lymphopenia in IC animals and 0% in BC animals corresponds well with the 25% IC and 0% BC prevalence predicted for the inheritance of a trait determined by a single autosomal recessive gene with 100% penetrance.

We also had the opportunity to examine the T lymphocytes of diabetic animals (blackened symbols in Fig. 1). All diabetic animals (11/11) were lymphopenic ($p = 2 \times 10^{-7}$). In our experience, no nonlymphopenic rat has ever developed spontaneous diabetes mellitus, and lymphopenia is present from the neonatal period onward (unpublished observation).

The T cell lymphopenia described was also evident using the OX19 ("panT") and OX8 ("suppressor/cytotoxic") T cell markers (Fig. 2). Antibody W3/13 recognizes a cell surface antigen that is present not only on T cells, but also on large granular lymphocytes and polymorphonuclear cells (10). Because of its broader spectrum, W3/13 does not reveal the T cell lymphopenia as well as the

TABLE I

Generation	Wistar Furth			Brown Norway			Lewis			Summary		
	No.	No. DM	%DM	No.	No. DM	%DM	No.	No. DM	%DM	No.	No. DM	%DM
F1	106	0	0	118	0	0	124	0	0	348	0	0
F2	251	6	2.4	949	18	1.9	351	8	2.3	1,551	32	2.1
1 BC	25	0	0	49	0	0	19	0		93	0	0
1 IC	535	7	1.3	533	7	1.3	177	7	4.0	1,245	21	1.7
2 BC	76	0	0	31	0	0	0	0	0	107	0	0
2 IC	694	7	1.0	106	2	1.9	0	0	0	800	9	1.1
3 BC	89	0	0	13	0	0	0	0	0	102	0	0
3 IC	657	9	1.4	0	0	0	0	0	0	657	9	1.4
4 BC	62	0	0	0	0	0	0	0	0	62	0	0
ΣF1, ALL BC	358	0	0	211	0	0	143	0	0	712	0	0
ΣF2, ALL IC	2,137	29	1.4	1,588	27	1.7	528	15	2.8	4,253	71	1.7

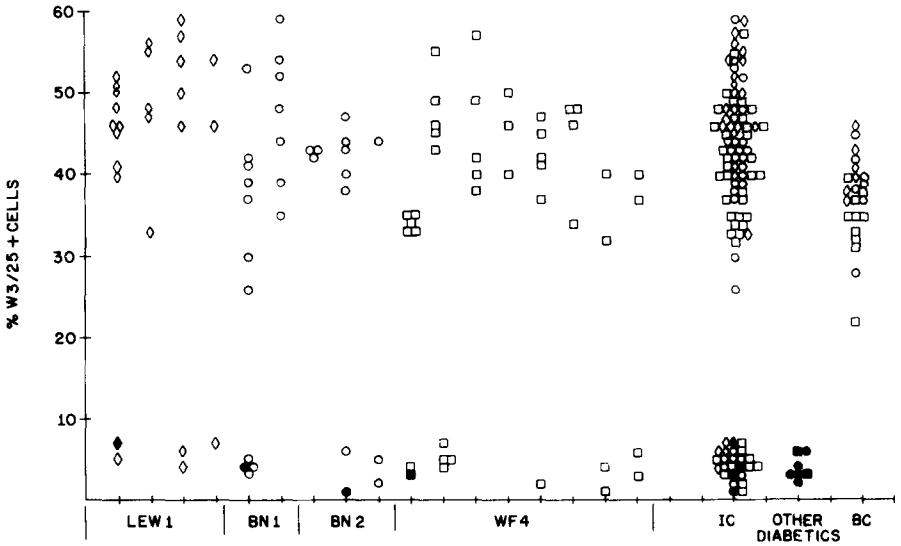

FIGURE 1. Percentage of W3/25 positive cells were studied in backcrosses and intercrosses of Lewis (◇), Brown Norway (○), and Wistar-Furth (□) rats. Darkened symbols denote diabetic animals. Littermates are represented as columns. W3/25 positive cells were determined in IC1 Lewis rats (LEW 1), IC1 Brown Norway (BN 1), IC2 Brown Norway (BN 2), and IC4 Wistar-Furth (WF 4). *IC* column represents all intercross litters studied. *OTHER DIABETIC* represents diabetic animals whose littermates were not studied. *BC* column represents the percentage of W3/25+ cells in backcross animals.

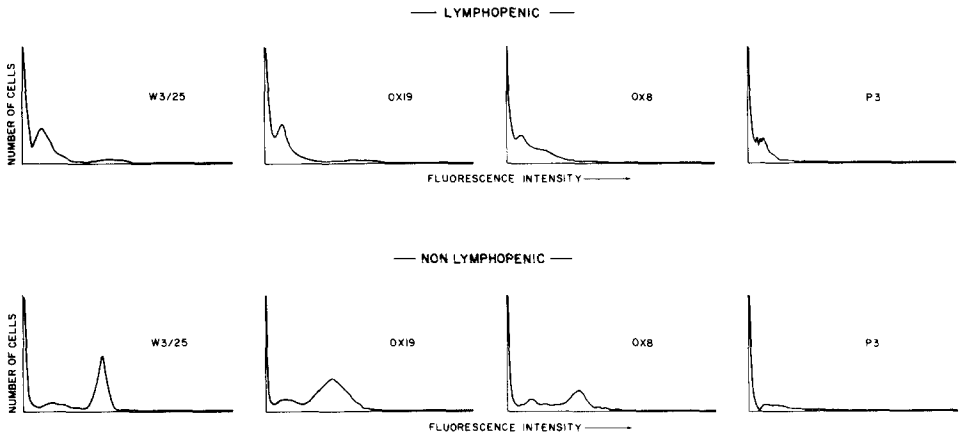

FIGURE 2. Fluorescence histograms on two IC rats, one with and one without T cell lymphopenia, using monoclonal antibodies recognizing different T cell subsets. P3 is a negative control. *X*-axis, log integral green fluorescence; *Y*-axis, cell number.

other antibodies tested. Occasional lymphopenic animals have percentages of W3/13+ cells that are within our normal range although their OX19+ cells are greatly decreased (Fig. 3). In two-parameter fluorescent flow cytometer patterns (Fig. 4), it can be seen that the size distribution (as determined by forward angle light scatter) of the W3/13+ cells is different between lymphopenic and normal rats. In the lymphopenic animals, W3/13+ cells are OX19 and W3/25 negative,

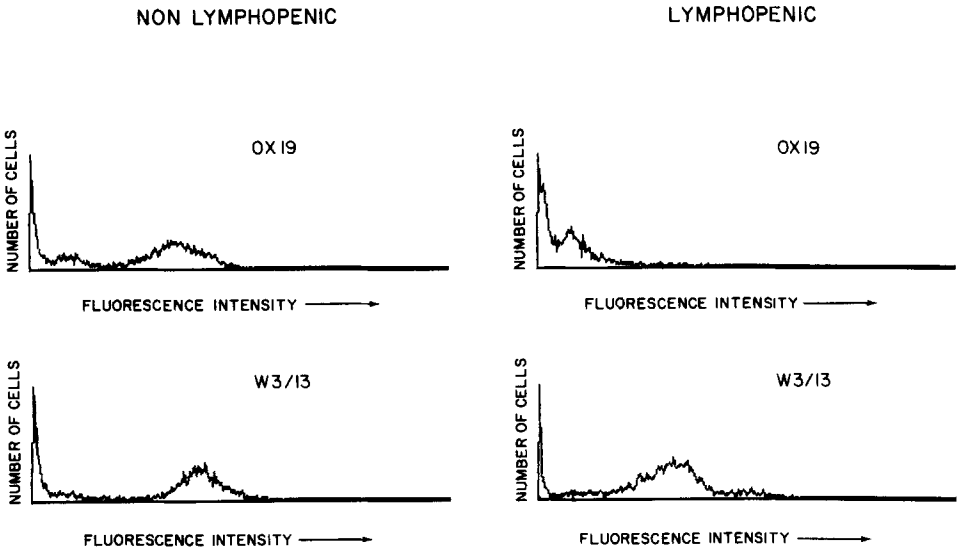

FIGURE 3. Fluorescence histograms on two IC rats, one with and one without T cell lymphopenia using monoclonal antibodies OX19 (T cell specific antibody) and W3/13 (T cells and some non–T cells).

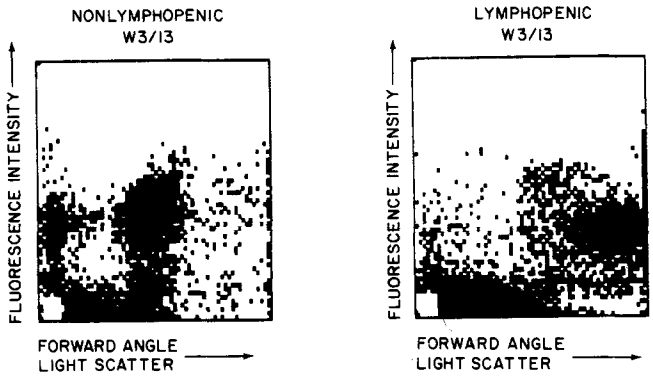

FIGURE 4. Two-parameter histograms on two IC rats, one with and one without T cell lymphopenia. The *X*-axis represents forward angle light scatter which increases with increasing cell size. Mononuclear cells show greater forward angle light scatter than erythrocyte and noncellular debris which is near the origin. The *Y*-axis represents log intergral green fluorescence. OX19 is a T cell specific antibody, W3/13 reacts with T and some non–T cells.

whereas in the nonlymphopenic rats the great majority are T cells (OX19+, W3/25+).

The rat major histocompatibility complex is termed RT1. The BB rat shares the $RT1^u$ type with WF rats, and differs from BN ($RT1^n$) and L ($RT1^l$) rats. Monoclonal antibodies reacting with the $RT1^u$, $RT1^l$, and $RT1^n$ class I alloantigens were used to follow the inheritance of BB $RT1^u$ genes among L and BN intercrosses. Among diabetic animals eight of nine were homozygous for $RT1^u$, and one was a u/n heterozygote. All 19 BC animals studied (19/19) were heterozygotes (u/n or u/l), indicating that their diabetic parents expressed at least one u haplotype. Lymphopenia was independent of RT1 haplotype.

Discussion

The breeding used in this study was designed to transfer the genes necessary for the expression of diabetes mellitus in the BB rat to several inbred rat strains. During the breeding, animals were selected only on the basis of overt diabetes, without knowledge of lymphopenia or RT1 phenotype. As a first approximation, the genome that is not linked to genes involved in the diabetic process undergoes serial 1:1 dilutions by repeated backcrossing. Intercrossing merely allows for the expression of recessive genes. This type of breeding protocol is particularly powerful for genetic studies because of its straightforward nature and its inherent positive and negative selection pressures. Using these animals, we have found that there are at least two independent genes needed for the expression of the diabetic phenotype in BB rats.

The T cell lymphocytopenia of BB rats has now been described by many groups around the world (11). Its importance to the diabetic syndriome in BB's can be inferred from the fact that replacement therapies such as bone marrow transplantation and mononuclear cell transfusion can prevent the diabetes (12, 13). Our data indicate that the lymphopenia is inherited by simple autosomal recessive genetics. No BC animals exhibit lymphopenia, but 24% of intercrosses do. Furthermore, 11 of 11 diabetic animals were lymphopenic, supporting our hypothesis that the T cell lymphocytopenia is absolutely required for the development of diabetes ($p = 2 \times 10^{-7}$).

We also determined the RT1 haplotype of the L and BN IC rats. There was no association between RT1 type and lymphopenia. However, eight of nine diabetic animals were homozygous for the BB's $RT1^u$ and the other was a u/n heterozygote. This association beteen RT1 type and the expression of diabetes is strengthened by its presence in animals that have undergone three backcrosses. Among diabetic animals, the 8:1 excess of RT1 homozygotes over heterozygotes suggests that the probability of an animal becoming overtly diabetic is increased by having two RT1-DM genes, though an occasional RT1-DM heterozygote can also develop diabetes. This would be analogous to experimental allergic encephalomyelitis in which susceptibility and severity of this disease is linked to RT1 so that animals homozygous for Ir-EAE develop more severe disease (14). Alternatively, RT1-DM could be a strictly recessive gene and there has been a crossover between the RT1-DM site and the RT1.A locus in the one diabetic u/n heterozygote. The lack of a higher percentage of diabetes among WF ($RT1^u$) intercrosses, when compared with L and BN intercrosses, suggests that the RT1-DM gene linked to the $RT1^u$ of BB rats is not present in WF animals, though they share the same serologically defined class I antigens. The linkage of susceptibility to induction of autoimmune endocrine disease with the MHC region has been established in the mouse, where a point mutation in the H-2 region can result in conversion from disease resistance to susceptibility (15).

This study is in general agreement with the findings of Dr. Colle and coworkers (6–8) in matings of BB rats with either Buffalo or Lewis rats. They used a very different breeding program (following sequential generations rather than backcross and intercross) and the non–T cell specific monoclonal antibody W3/13 to examine lymphopenia. Though their studies reveal a trend towards a recessive lymphopenia gene related to diabetes and independent of RT1 inher-

itance, the genetic relationships were complicated by the development of diabetes in 1 of 11 BB × Buffalo F1 animals (7). We found no diabetic animals in 348 F1 animals nor in 364 backcrosses. In the Montreal study, lymphopenia was associated with diabetes using the W3/13 marker, but the percentage of W3/13+ cells was 10–35% in lymphopenic animals (7), greatly exceeding what we find with OX19 or W3/25. Additionally, in their studies lymphopenia is variable in its age of development and was not present in 2/12 diabetic rats before the diagnosis of diabetes. This difference is probably due to the use of antibody W3/13, which lacks the T cell specificity of antibodies OX19, W3/25, and OX8 in peripheral blood (Figs. 3 and 4).

In breeding studies of an early colony of BB/W rats, Like and co-workers (3) concluded that "diabetes is transmitted as an autosomal recessive gene," but felt that "the data do not rule out the possibility that more than one gene is involved in the syndrome's transmission." We suspect that one of the alleles segregating in their early colony was associated with normal T lymphocyte numbers; that is, all of their early BB/W rats were not lymphopenic, though they may all have carried RT1-DM. If this were the case, then diabetes susceptibility would be present only in lymphopenic animals, and would follow an autosomal recessive inheritance pattern.

The finding that at least two separate genes are absolutely required for expression of diabetes in this BB rat colony, one gene (*l*) producing an easily recognized phenotype, should aid in the search for specific diabetogenic gene products in these animals.

Summary

The BB rat develops a syndrome of autoimmune diabetes similar to Type I diabetes of man. It also has a severe T cell lymphopenia. As part of an ongoing breeding program to transfer the diabetogenic genes of the BB rat onto inbred rat strain backgrounds, diabetic animals were used in a backcross (BC)-intercross (IC)-backcross breeding scheme with Brown Norway (BN), Lewis (L), and Wistar-Furth (WF) inbred rats. We have used monoclonal antibodies to analyze both lymphopenia and major histocompatibility (MHC) antigens (the RT1 locus in the rat) in relation to the development of diabetes. To examine T cell subsets we used a panel of monoclonal antibodies, in particular W3/25 and OX19, which discriminate the abnormal phenotype better than W3/13. In our breeding program, at least two independent genes or gene complexes are required for the expression of diabetes. One gene determines the lymphopenia, is inherited by simple autosomal recessive genetics and is not linked to the MHC. The second gene is linked to the MHC. Both genes are necessary, but neither gene is sufficient by itself for the development of diabetes.

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