Brief Definitive Report

SPONTANEOUS DIABETES MELLITUS SYNDROME IN THE RAT

I. Association with the Major Histocompatibility Complex*

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There is a well-known genetic predisposition to the development of insulin-dependent diabetes mellitus in man. Current information indicates that susceptibility genes are linked to the major histocompatibility complex (MHC), human leukocyte antigen (HLA), and specifically to the D/DR locus of this region (1). However, the presence of these susceptibility genes is not in itself sufficient to result in the clinical manifestation of the disease, and additional factors (either genetic or environmental) are necessary for the expression of the disease. Because of the relative rarity of IDD and its usual occurrence as a single event within families, experiments to test hypotheses concerning the mode of transmission and pathogenesis of the disease are difficult to design in man.

The BB Wistar rat is a promising animal model for such studies. This strain develops an acute form of spontaneous diabetes mellitus characterized by hyperglycemia, low-serum insulin levels, ketonuria, and weight loss (2). Examination of the pancreas reveals decreased insulin content, degranulation, and necrosis of beta cells in the islets of Langerhans, and, in the early and preclinical phase of the disease, insulitis, i.e., lymphocytic infiltration of the islets (3). The histopathologic findings are similar to that described in the early stages of juvenile diabetes mellitus (4).

It is not known whether susceptibility (or resistance) in this rat model is linked to the MHC of the rat or the RT1 locus. Here we report studies designed to test the hypothesis that the clinically apparent spontaneous diabetic syndrome is associated with the RT1 complex of the rat.

Materials and Methods

Two male BB rats were obtained from the breeding colony maintained by the Health Protection Branch of the Department of Health and Welfare of the Government of Canada. They were bred to two highly inbred Lewis (LEW, RTL 1/1) females obtained commercially (Microbiological Associates, Walkersville, Md.). Eight (BB × LEW) F_1 males and females were then intercrossed to produce a population of eight F_2 litters of 89 progeny. After the detection of the first spontaneously diabetic rat in the F_2 population, the F_1 mating pair that had produced this animal was remated to produce three additional litters of 39 progeny, a total of 48 offspring. All animals were weighed three times a week and when weight decreased or failed to increase between weighing periods, urine was tested for glucose and ketones using Dextrostix

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(Ames Co., Elkart, Ind.). When glucosuria was found, plasma glucose was determined using an autoanalyzer (Beckman Instruments, Inc., Fullerton, Calif.) (glucose oxidase method).

Sera used for erythrocyte (RBC) typing was prepared by hyperimmunization of appropriate rat strains with three skin grafts and, after absorption where indicated, was shown to be monospecific by typing erythrocytes of a reference panel of other inbred lines maintained in this laboratory. All animals were serotyped between 40 and 60 d of age by a Ficoll hemagglutination technique (5) for determination of the A region haplotype of RT1. After washing the individual RBC in 0.15 M NaCl-Hepes, pH 7.2, a 0.4% cell suspension in buffer was made and 100 μl was added to 100 μl of the particular antiserum diluted in 5% Ficoll. The reactants were incubated for at least 20 min at 20°C and then centrifuged at 700 *g* for 1 min. A droplet of the mixture was read microscopically and scored positive or negative. Appropriate control RBC were used each time. After having determined the A region haplotype of the BB strain, mixed lymphocyte cultures of peripheral blood were performed to establish the B region haplotype of the BB strain using a dextran-citrate lymphocyte isolation technique previously described (6, 7). Peripheral blood lymphocytes were obtained by sedimentation in 10% dextran plus 2.5% sodium citrate. Cells at a concentration of 1×10^6/ml suspended in minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) and 10% fresh BN or LBN F_1 hybrid serum. The appropriate mixtures of cells as well as controls were incubated for 6 d at 37°C in an atmosphere of 5% CO_2 and 95% air. Proliferation was quantitated by the addition of 1 μCi of ^{125}I (Amersham-Searle Ltd., Toronto, Ontario) 16 h before harvesting. The cell pellet was washed twice with normal saline and twice with 5% trichloroacetic acid, centrifuged at 800 *g*, and the precipitated radioactivity was counted for ^{125}I.

At the time of termination of the 120-d observation period, tissue from the tail of the pancreas was fixed in Bouin's solution and prepared for conventional light microscopic study. Step sections from each paraffin block were stained with hematoxylin-phloxine-saffron. In addition, these sections were each stained by the Grimelius silver nitrate technique for A cells (8) and Gomori aldehyde fuchsin technique for B cells (9). All histologic sections were examined without prior knowledge of the genetic characterization of the experimental animals. An additional sample from the tail of the pancreas was excised, trimmed and blotted, weighed, and immediately placed in 2 N acetic acid, boiled for 10 min, homogenized, and centrifuged. Aliquots of the supernate were diluted with phosphate buffer, pH 7.4, and assayed for insulin and glucagon. Acetic acid extracts were neutralized with 0.1 N NaOH before assay. Immunoreactive insulin was assayed using a dextran-coated charcoal method (10) as previously described (11). Purified crystalline rat insulin (courtesy of Dr. R. Chance, Eli Lilly Co., Indianapolis, Ind.) was used for iodination by a chloramine T method (12) and for reference standards. ^{125}I was purchased from Amersham Corp., Oakville, Ont. Immunoreactive glucagon was assayed using antisera designated 30 K and purchased from the University of Texas at Dallas. Porcine glucagon from Nova Research Institute (Bagsvaerd, Denmark) was used as reference standard and for iodination by a chloramine T method (12). The iodinated peptide was purified on a QAE-25 column (Pharmacia Fine Chemicals, Piscataway, N. J.), 15- × 30-cm, eluted with buffer containing 9.69 g Tris (Hydroxymethyl) aminomethane, 208 ml of 0.1 N HCl, 392 ml distilled water, and 4.63 g NaCl (pH 8.6) with 1% bovine serum albumin and 50 KIU Trasylol (FBA Pharmaceut. Inc., New York) for each milliliter of buffer. The purification was carried out at 4°C and the eluates were collected into tubes containing 0.1 ml Trasylol each. The radioimmunoassay was performed according to the protocol accompanying the antiserum (Dr. R. Unger, University of Texas, Dallas).

Statistical analysis was done using the two-way analysis of variance technique from the Statistical Package for the Social Sciences ANOVA program (13) run on the Montreal Childrens Hospital Research Institute computer (3000; Hewlett-Packard Co., Palo Alto, Calif.).

Results

The BB Wistar rats obtained from the Ottawa colony showed serotyping reactions that indicate that they are of the u/u genotype at the A region of RT1. Mixed lymphocyte culture reactions reveal that they and their F_2 progeny who serotyped u/u at the A region were also u/u at the RT1 B region by virtue of a negative two-way

reaction with the Wistar-Furth (WF, $RT1^u$) standard reference strain and a positive two-way reaction in the culture sets using the ACI ($RT1^a$) strain (Table I).

The (BB × LEW)F_2 hybrids produced from eight mating pairs were examined for segregation of the RT1 haplotypes and the segregation did not depart from the expected 1:2:1 ratio. The sex ratio was also as expected (Table II). In the four litters from the mating pair producing the spontaneously diabetic animals, there was a distortion of the expected 1:2:1 ratio, which resulted in an increased number of animals homozygous for the u haplotype.

None of the 23 F_1 hybrids developed spontaneous diabetes mellitus (SDM). 4 of the 128 F_2 animals developed SDM; details are presented in Table III. All animals that developed SDM were homozygous u/u. These four all came from a single breeding pair producing four litters of 48 progeny. Statistical analysis of u/u vs. non-u/u progeny gave a Fisher's exact probability of $P = 0.03$. The age at onset of the clinical disease was similar to that found in the BB colony.

The insulin concentration of the pancreatic tail from the nondiabetic animals in the first eight litters obtained by random mating of the F_1 is shown in Table IV. (The last three litters from the mating pair that had produced the diabetic animals were used for further mating and are excluded from analysis because many are still alive and others were killed at a more advanced age.) Pancreatic insulin concentration in the two diabetic animals surviving from the first eight litters were also excluded from analysis. There was wide variation of the insulin concentration in the tail of the pancreas. Two-way analysis of variance revealed significant differences between litters ($F = 11.07$, $P < 0.001$) and between haplotypes ($F = 5.45$, $P < 0.007$). Glucagon concentrations were less variable with litter differences contributing significantly to total variance; no effect of haplotype was seen.

Histologic examination of the pancreas was conducted in 89 of the 124 nondiabetic F_2 animals (pancreatic specimens from the first two litters were not examined) without knowledge of the haplotype data. No insulitis was observed in these animals. However, periductal and/or multifocal acinar lymphocytic infiltration were noted in 8 of 81 animals as well as in the 2 autopsied diabetic rats. Four of these animals were RT1 u/u and four were RT1 u/l. Rats with the l/l haplotype were not observed to have any pancreatic lesions.

Discussion

The BB rat is a Wistar-derived strain that spontaneously develops an insulinopenic hyperglycemia and ketosis in association with lymphocytic infiltration of pancreatic

TABLE I

RT1 B Region Haplotype Responses of BB and (LEW × BB) F_2 Rats

	^{125}IUdR uptake (cpm ± SEM)		
	Unstimulated	vs. WF	vs. ACI
BB overtly diabetic	104 ± 22	63 ± 12	1,325 ± 62
F_2 hybrid typing u/u at RT1 A region by hemagglutination	64 ± 13	41 ± 9	2,834 ± 285
		119 ± 55	4,310 ± 32
	91 ± 9	145 ± 27	257 ± 83
	53 ± 2	89 ± 4	2,972 ± 39
WF ($RT1^u$)	72 ± 30		3,971 ± 257
ACI ($RT1^a$)	74 ± 16		

TABLE II

(BB × LEW) F_2 Haplotype and Sex Ratios from Eight Mating Pairs

Sex	RT1 genotype (A region)				Comparison expected	
	uu	ul	ll	Total	χ^2	P
Male	8	23	13	44		
Female	10	23	12	45		
Total	18	46	25	89	0.939	NS
	F_2 animals from a single pair producing diabetic progeny*					
Male	12	10	5	27	4.19	<.05
Female	9	5	7	21	6.11	<.025
Total	21	15	12	48	9.03	.01

* Also included in data above are the primary litter of nine rats from this sibship, two uu, five ul, two ll.

TABLE III

Characteristics of Spontaneously Diabetic Animals

Sex	RT1 haplotype	Age at onset	Insulin	Course
		d		
Male	u/u	55	2–3 U daily	Died at 120 d; pneumonia at autopsy
Female	u/u	80	2 U	Developed fulminant course 2 d post-partum; died 2 d later
Male	u/u	101	—	Weight loss from day 89 to day 103 with glucosuria for 2 d; recovery without insulin, presently alive
Male	u/u	73	3–3.5 U	Presently alive and requiring insulin

TABLE IV

Insulin and Glucagon Content of Pancreatic Samples of Nondiabetic F_2 Rats

Litter	Number	Insulin (mean ± SEM)	Glucagon (mean ± SEM)
		ng/mg wet:wt	*ng/mg wet:wt*
1	8	24.6 ± 7.3	4.0 ± 0.89
2	12	40.6 ± 7.3	5.4 ± 0.67
3	10	25.9 ± 9.7	2.7 ± 0.33
4	12	91.1 ± 13.4	3.2 ± 0.53
5	10	50.2 ± 11.3	2.8 ± 0.39
6	5	83.4 ± 26.8*	3.0 ± 0.92
7	11	96.5 ± 20.0	3.8 ± 0.45
8	13	141.9 ± 16.4	5.9 ± 1.20
Haplotype			
uu	16	80.4 ± 13.9	3.7 ± 0.43
ul	43	70.4 ± 10.3	3.7 ± 0.31
ll	22	87.9 ± 12.5	5.0 ± 0.82
Overall mean		73.3 ± 6.7	4.0 ± 0.28
Two-way analysis of variance			
		F for litter = 11.06 $P < .001$	F for litter = 2.67 $P < .018$
		F for haplotype = 5.45 $P < .007$	F for haplotype = 1.85 P = NS

* Excludes two animals that were diabetic.

islet tissue between 40 and 120 d of age. An acute form requiring insulin treatment, a stable form with failure to gain weight, but in which exogenous insulin is not essential for life, and a rare form of transient, apparently reversible disease have also been observed (2, 14). Once the diabetic syndrome is fully developed, progressive destruction of B cells occurs, leading to an "end-stage" lesion characterized by small

islets containing virtually no B cells. Insulitis has also been observed in rats not clinically diabetic.[1]

Similarities between SDM in the BB rat and human insulin-dependent diabetes in which there is a strong association with gene products coded for by the human MHC, HLA, make this an interesting model. We first determined that the BB Wistar rat showed typing reactions indicating the u/u haplotype at both the A region (analogous to the human A and B loci) and the B region (analogous to the human D/DR locus). In the present study, we produced F_2 hybrids by intercrossing F_1 hybrids produced from BB males (RT1 u/u) and LEW females (RT1 l/l). Four cases of spontaneous diabetes resulted; three cases were severe and required insulin treatment and one was transient. All of the affected animals were homozygous RT1 u/u, suggesting that, as in the human, the susceptibility is associated with gene products of the MHC. However, the number of disease events observed is so small that it is not possible to conclude that homozygosity at u/u, excluding u/l progeny, is a necessary condition. As in man, not all of the siblings sharing the same haplotype became overtly diabetic, nor was insulitis present in the litter mates of the diabetic animals when the latter were examined at ages >120 d of age. However, pancreatic periductal and acinar lymphocytic infiltration were seen in eight nondiabetic animals and in both of the autopsied diabetic rats. This type of lesion was not seen in any RT1 l/l animals; however, this is not a statistically significant finding. Similar lesions have been seen in human cases of insulin-dependent diabetes (4).

Insulin concentration (Table III) varied widely among the animals. Previous studies from our laboratory have indicated that in the symptomatic diabetic animals requiring insulin for maintenance of body weight and prevention of ketosis, insulin concentrations in the tail of the pancreas were generally <1 ng/mg wet wt of tissue. However, variability in insulin concentrations was seen in litter mates of BB rats and in control animals (animals bred for several generations with the appearance of the SDM syndrome) from the same colony.[1] Differences between litters account for most of the variance, but differences between haplotypes also contribute significantly to total variance. It is possible that the reduced insulin concentrations reflect destruction of some islets that was not sufficiently extensive to cause clinical disease or to be evident on histologic examination. Alternatively, it is possible that there are individual differences in the islet cell mass that determine the ability of an animal to withstand the injury responsible for B cell destruction. Because differences in glucagon concentration in the same specimens were not as marked, we concluded that the changes noted in insulin concentration are not due to sampling variation. Persistence of normal pancreatic concentrations of glucagon in these animals point to noninvolvement of the A cells of the islet of Langerhans in the primary pathogenesis of the syndrome.

Summary

A syndrome of spontaneous diabetes mellitus has been previously described in a partially inbred rat strain called BB Wistar. We have determined whether there is major histocompatibility complex (MHC) linkage as well as other predisposing

[1] Tannenbaum, G. S., E. Colle, and T. A. Seemayer. Time course studies in the BB Wistar rat. Manuscript submitted for publication.

haplotype-associated factors of development. BB rats are RT1 (MHC) genotype u/u. Using BB × Lewis F_1 hybrid matings, an F_2 study analyzed 128 rats from 8 primary and 3 additional litters from a breeding pair producing a diabetic offspring. 4 of 128 F_2 rats, all from the 48 progeny of same breeding pair, became clinically diabetic. The four diabetics were all genotype u/u ($P = 0.03$). In the primary F_2 litters, haplotype distribution was not different from the 1:2:1 expected ratio. However, in the four litters from the F_1 breeding pair producing diabetics, there was an increased number of u/u animals. Two-way analysis of variance revealed significant differences in pancreatic insulin content between litters (diabetics excluded), $P < 0.001$, and between haplotypes $P < 0.007$ with heterozygous u/l < u/u < l/l progeny. The glucagon content showed no significant differences. These data demonstrate (*a*) MHC linkage with spontaneous diabetes in this rat model; (*b*) penetrance similar to the human disease; and (*c*) a possible association of MHC haplotype with pancreatic inflammation as well as insulin content in nondiabetic F_2 siblings.

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References

1. Christy, M., A. Green, B. Christau, M. Kranman, J. Nerup, P. Platz, M. Thomsen, L. Ryde, and A. Svejgaard. 1978. Studies of the HLA system and insulin dependent diabetes. *Diabetes Care.* **2:**209.
2. Nakhooda, A. F., A. A. Like, C. I. Chappel, F. T. Murray, and E. B. Marliss. 1977. The spontaneously diabetic Wistar rat. *Diabetes.* **26:**100.
3. Seemayer, T. A., L. L. Oligny, G. S. Tannenbaum, H. Goldman, and E. Colle. 1980. Animal model: spontaneous diabetes mellitus in the BB Wistar rat. *Am. J. Pathol.* **101:**485.
4. Gepts, W. 1965. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes.* **14:**619.
5. Kunz, H. W., and T. J. Gill, III. 1974. Genetic studies in inbred rats. I. Two new histocompatibility alleles. *J. Immunogenet.* **1:**413.
6. Wilson, D. B. 1967. Quantitative studies on the mixed lymphocyte interation in rats. I. Conditions and parameters of response. *J. Exp. Med.* **126:**625.
7. Guttmann, R. D. 1974. Membrane properties and functional activity of lymphocytes from cyclophosphamide pretreated rats. *J. Immunol.* **112:**1594.
8. Grimelius, L. 1968. A silver nitrate for a2 cells in human pancreatic islets. *Acta Soc. Med. Upsal.* **73:**243.
9. Gomori, G. 1950. Aldehyde fuchsin: a new stain for elastic tissue. *Am. J. Clin. Pathol.* **20:** 665.
10. Herbert, V., K. D. Lau, C. W. Gottlieb, and S. J. Bleicher. 1965. Coated charcoal immunoassay of insulin. *J. Clin. Endocrinol. Metab.* **25:**1375.
11. Goldman, H., and E. Colle. 1976. Human pancreatic islets in culture: effects of supplementing the medium with homologous and heterologous serum. *Science* (*Wash. D. C.*). **192:** 1014.
12. Greenwood, F. C., W. H. Hunter, and J. S. Glover. 1963. The preparation of ^{131}I-labeled human growth hormone of high specific activity. *Biochem. J.* **89:**114.
13. Nie, N. H., C. H. Hull, J. G. Jenkins, K. Steinbrenner, and D. H. Bent. 1975. Statistical Package for the Social Sciences. 2nd edition. McGraw-Hill, New York. 398.
14. Nakhooda, A. F., A. A. Like, C. J. Chappel, C. N. Wei, and E. B. Marliss. 1978. The spontaneously diabetic Wistar rat (the BB rat). *Diabetalogia.* **14:**199.