BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **239,** 106–110 (1997)
ARTICLE NO. RC977436

Phenotypic Consequences after Restoration of Lymphopenia in the Diabetes-Prone BB/OK Rat

Ingrid Klöting, Peter Kovacs, and Beate Kuttler*
*Department of Laboratory Animal Science at the *Institute of Pathophysiology, University of Greifswald, D-17495 Karlsburg, Germany*

Received September 1, 1997

Besides other factors, lymphopenia (*ll*) is essential for development of insulin-dependent diabetes mellitus in the BB rat. It is unknown which phenotypic consequence may have a non-lymphopenia. Therefore, the region containing the lymphopenia gene of the BB/OK rat was replaced with that of the non-lymphopenic (LL) and hypertensive rat, SHR (11 cM, *D4Mit6-LL-Npy-Spr*). The resulting congenic strain, BB.LL, did not develop diabetes up to an age of 30 weeks and was non-T-lymphopenic. The SHR region did not influence the blood pressure of the BB.LL rat, but influenced obviously the motor activity, body weight, serum lipids, and the 24h urine excretion of urea, sodium, and potassium.

Key Words: **rats; congenic; T-lymphocytes; blood pressure; lipids; gene interaction.**

The diabetes-prone BB rat is a well-established animal model of insulin-dependent diabetes mellitus. The animals develop an autoimmune diabetes closely resembling human disease including rapid onset, autoimmune destruction of pancreatic islet cells, autoantibodies to ß cell components and the requirement for daily injections of insulin. Similarly to human beings the class II genes of MHC ($RT1^u$ haplotype) are associated with diabetes development and explain some, but not all of the inherited predisposition to the disease in the BB rat (1). Despite close resemblance there is also a marked difference to human diabetes. The BB rat is characterized by an immune deficiency which finds expression in a profound T- lymphopenia which is not observed in human diabetics. The lymphopenia is inherited as an autosomal recessive trait (*ll*) and has been mapped on chromosome 4 between the loci *D4Mit6* and *Npy* (2). By several crossing studies it has been demonstrated that beside MHC class-II genes of the $RT1^u$ haplotype the lymphopenia is essential for diabetes development in the BB rat (3-6). Up to now it is unknown which consequence has the restoration of lymphopenia for the phenotype of BB rats. The lymphopenia gene is unknown so that only the generation of a non-lymphopenic congenic BB rat strain gives an opportunity to get an answer. By this procedure the chromosomal region of the BB rat carrying the lymphopenia gene (*ll*) has to be exchanged by a region of a non-lymphopenic (*LL*) rat strain. Congenic strains are usually derived by backcross breeding and genomic selection techniques, by which a specific chromosomal region is transferred from the strain A onto the genetic background of a recipient strain B designated B.A. If such congenic B.A strain shows a difference to the progenitor strain B, one can conclude that there is a locus within the transferred chromosomal region affecting the appropriate trait.

Because only BB rats have so far been shown to be lymphopenic each available inbred rat strain could be used as chromosomal donor. We have chosen the non-lymphopenic (LL) and spontaneously hypertensive rat, SHR/Mol, for the diabetic BB rat differs from human diabetics not only in the lymphopenia but also in the development of diabetic complication. In contrast to human diabetics the diabetic BB rat does not develop hypertension and nephropathy markedly determining the morbitity and mortality of diabetic patients. One supposes that the BB rat is not genetically susceptible for these diseases (7-9). Therefore, we used the spontaneously hypertensive and non-lymphopenic SHR rat as chromosomal donor strain, since around the *Npy* locus on chromosome 4 a quantitative trait locus (QTL) for blood pressure regulation was detected in a segregating population of SHR and normotensive WKY rats (10). With the aid of this chromosomal donor strain the possibility should be given not only to create a non-lymphopenic congenic BB rat strain but also to study the physiologic importance of this putative blood pressure QTL onto the genetic background of the normotensive BB/OK rat. For this purpose commercially available hypertensive SHR/Mol and our own well-characterized diabetic BB/OK rats (11) were crossed and the resulting cross hybrids were repeatedly backcrossed onto dia-

0006-291X/97 $25.00

betic BB/OK rats as described recently (12). By this procedure a region of the SHR/Mol rat spanning about 11 cM and comprising the loci *D4Mit6, LL, Npy* and *Spr* was transferred onto the genetic background of BB/OK rats. The congenic strain was designated BB.LL. Animals of this newly established strain were phenotypically characterized for lymphopenia, diabetes occurrence, hypertension- and diabetes-related traits.

MATERIAL AND METHODS

Animals. BB.LL rats were generated by cross of BB/OK and SHR/Mol rats. The resulting cross hybrids were repeatedly backcrossed onto diabetic BB/OK rats. To accelerate the generation of congenic BB.LL rats we selected animals which were heterozygous for *D4Mit6, Npy* and *Spr* and homozygous for BB alleles at most of the 72 background loci examined. After 5 backcross generations the animals were already homozygous at background loci and were intercrossed. Animals homozygous for SHR alleles at the loci of interest were selected and founded the congenic BB.LL strain. The genetic background of BB/OK rats was confirmed by testing of BB.LL rats (F1) with additional 86 microsatellite markers which were not tested during creation of these congenics.

All BB/OK (F40, 11) and BB.LL rats (N6 F2) used were bred and kept in our own animal facility under strict hygienic conditions and were free of major pathogens as described elsewhere (11). They were given a laboratory diet (Altromin, Lage, FRG) and water ad libitum. The animals were kept with a rhythm of 12h light (5 a.m. to 5 p.a.) : 12h dark and housed 3 per cage (Size 3, Ehret GmbH, Emmendingen, FRG).

Lymphocyte preparation. Lymphocytes of nondiabetic male and female BB/OK (n=8) and BB.LL rats (n=8) were prepared from the peripheral blood by Ficoll/Visotrast gradient centrifugation as described earlier (13). The number of cells was adjusted to 1×10^6 per ml PBS containing 1% fetal calf serum and 0.1% NaN_3. Viability was >98% as ascertained by vital staining with acridine orange (Boehringer Mannheim GmbH, FRG) and ethidium bromide (Sigma Chemical Co., St Louis, MO) on a fluorescence microscope.

Lymphocyte phenotyping. Peripheral blood cell phenotypes were determined by means of flow cytometry as described elsewhere (14). The following primary mAb recognizing T-cells [1F4 (CD3), kindly provided by T. Tanaka, University of Tokyo, Japan; R73-FITC ($\alpha\beta$TCR), kindly provided by T. Hünig, University of Würzburg, FRG], resting, mature T-cells [3G2 (RT6.1), kindly provided by R. Schwinzer, Medizinische Hochschule Hannover,FRG], B-Lymphocytes [OX33-FITC (CD45RA/AB), Pharmingen Deutschland GmbH], NK-cells [10/78, kindly provided by T. Hünig, University of Würzburg, FRG], T_H-cells [W3/25-FITC (CD4), Serotec Camon Labor-Service GmbH, FRG] and $T_{S/C}$-cells [341.6-FITC (CD8β), Serotec Camon Labor-Service GmbH, FRG] were used. The cells were analyzed on a Coulter Profile II Cytofluorograph (Hialeah, USA).

Diabetes occurrence. Diabetes occurrence was investigated in 3 complete litters of BB.LL (n=32) and BB/OK (n=36) up to an age of 30 weeks. All animals were screened for glucosuria twice a week by test tapes as described (11).

Telemetry. Five nondiabetic BB/OK and 3 BB.LL males at an age of 14 weeks were implanted with sensors (Data Sciences International, St. Paul, MN, USA) measuring telemetrically systolic (SBP, mm Hg) and diastolic blood pressure (DBP, mm Hg), heart rate (HR, beats/min) and motor activity (MA, movements/5min). One week after implantation of sensors the measurements were carried out every 5 min over a period of 5 days per animal.

Metabolic phenotyping. Nondiabetic males and females of BB/OK (10:6) and BB.LL (11 : 5) were studied at an age of 14 and 18 weeks for body weight, serum triglycerides, cholesterol, total protein, creatinine, urea, calcium and phosphate and 24 hr urine excretion of albumin, total protein, creatinine, urea, calcium, phosphate, sodium and potassium. All determinations were carried out with an automatic analyser (Roche Cobas Mira Plus, Roche, Switzerland). Blood samples were obtained by puncturing the ophthalmic venous plexus shortly before the animals were placed in metabolic cages. Urine samples were collected after housing the animals in metabolic cages for 24 hours.

Data analysis. Data were evaluated by using the statistical analysis system SPSS. The values are given as mean ± SD. Significant differences of mean values were checked by ANOVA analysis. Regarding the telemetric values, a single daytime mean and a single nighttime mean were calculated for each rat for 5 days and checked for significance. The values of serum and urine constituents measured at 14 and 18 weeks were calculated as mean value per animal.

TABLE 1

Number of Leucocytes and Percentage of B-Lymphocyte and T-Lymphocyte Phenotypes in Peripheral Blood of Nondiabetic BB/OK and BB.LL Rats

	BB/OK[a] n = 8	BB.LL n = 8
No. of leucocytes/μl	3827 ± 799	5888 ± 1071
Lymphocyte phenotypes (mAb)	%	%
B cells (OX33)	54.1 ± 4.6	17.9 ± 3.3
T cells (IF4)	14.4 ± 9.6	63.7 ± 13.3
T cell receptor (R73)	10.5 ± 2.0	66.4 ± 4.4
T_H cells (W3/25)	26.5 ± 5.6	54.8 ± 3.7
$T_{S/C}$ cells (341.6)	1.8 ± 1.7	19.2 ± 3.1
NK cells (10/78)	22.9 ± 8.6	2.9 ± 1.8
RT6.1 positive cells	0.5 ± 0.5	47.1 ± 4.2

[a] All values are significantly different between BB/OK and BB.LL at 0.01 percent level.

RESULTS

As shown in Table 1 the number of leucocytes, the percentage of T- lymphocyte subsets were significantly higher and those of B- and NK cells significantly lower in BB.LL than in BB/OK rats. These values of BB.LL rats were comparable with those of diabetes-resistant rat strains (14,15). Interestingly, although BB/OK rats are lacking RT6.1 positive cells they have been detected in BB.LL rats in a range determined in diabetes-resistant rats (15). Whereas 21 out of 36 BB/OK rats developed diabetes at a mean age of 126 ± 21 days all BB.LL rats remained normoglycemic up to an age of 30 weeks.

Table 2 summarizes the data of telemetric measurements. SBP, DBP and HR were comparable between BB.LL and BB/OK rats. MA was significantly increased in BB.LL compared with BB rats. Regarding the data in the light and dark phase the behaviour was comparable between BB/OK and BB.LL: higher values in the dark than in the light phase. Despite that, it is obvious that BB.LL rats were also active in the light phase (5 a.m. to 5 p.m.) as shown in Fig.1.

Both, BB.LL and BB/OK rats indicated also signifi-

TABLE 2

Systolic (SBP) and Diastolic Blood Pressure (DBP), Heart Rate (HR), and Motor Activity (MA) in Nondiabetic Male BB/OK and BB.LL Rats

	BB/OK n = 5	BB.LL n = 3
SBP (mmHg)	119.1 ± 5.3	117.5 ± 4.4
Light	117.6 ± 4.8	115.7 ± 4.5
Dark	120.8 ± 5.5	119.0 ± 4.4
DBP (mmHg)	88.1 ± 7.5	84.9 ± 5.2
Light	86.2 ± 7.3	82.3 ± 5.9
Dark	90.4 ± 7.9	87.3 ± 4.5
HR (beats/min)	353.0 ± 20.4	329.3 ± 6.7
Light	330.8 ± 24.8	308.7 ± 8.7
Dark	367.0 ± 16.9	350.0 ± 8.7
MA (movements/5 min)	19.7 ± 4.9[a]	30.4 ± 2.7
Light	9.6 ± 4.4[a]	19.7 ± 3.5
Dark	26.9 ± 6.6[a]	41.0 ± 5.2

[a] Significantly different between BB/OK and BB.LL rats at 5 percent level.

cant differences in body weight, serum and urine constituents summarized in Tables 3 and 4. The body weight was significantly increased in male and female BB.LL rats compared with BB/OK rats. Serum triglycerides, cholesterol, creatinine, urea and phosphate were also significantly higher in BB.LL than BB/OK rats. In the 24h urine excretion significantly elevated values were registered in urea, sodium and potassium whereas the 24h excretion of phosphate was significantly reduced in BB.LL rats in comparison with the parental strain BB/OK.

DISCUSSION

In contrast to studies in segregating populations, chromosome transfer studies in congenic strains can be used to isolate chromosome regions that contain known trait loci and to test directly their physiologic importance. Because we used hypertensive SHR rats as chromosomal donor we were able to investigate not only the physiologic importance of non-lymphopenia but also the importance of the blood pressure QTL on the genetic background of normotensive BB rats. As expected and measured in the peripheral blood the BB.LL rats were non-T-lymphopenic for the chromosomal region with the lymphopenia gene of the BB/OK rat was replaced by that of the non-lymphopenic SHR rat. The percentage of B- and T-lymphocyte subsets was comparable with those found in diabetes-resistant rat strains (14,15). Also the percentage of NK cells was in a range seen in normal rat strains, as in LEW rats (3.1 ± 0.3 %). An interesting finding was the high percentage of RT6.1 positive cells which was comparable with those of the SHR/Mol rat (47 ± 7 %). RT6 is a T-lymphocyte differenciation alloantigen and is expressed on mature

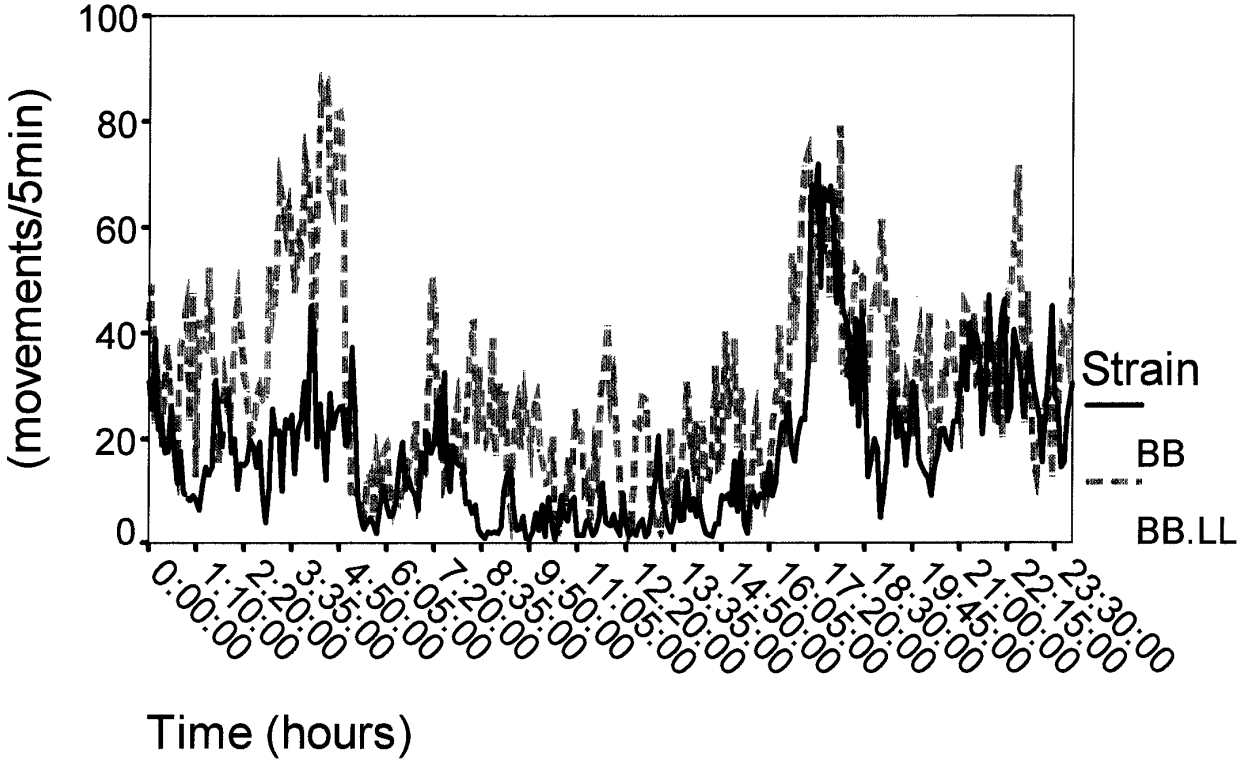

FIG. 1. Day profile of motor activity in nondiabetic male BB/OK and BB.LL rats.

TABLE 3

Body Weight and Serum Constituents in Male (M) and Female (F) Nondiabetic BB/OK and Congenic BB.LL Rats

No. of males:females	Sex	BB/OK 10:6	P value	BB.LL 11:5
Body weight (16 weeks)	M	341 ± 28[a]	<.01	403 ± 17[a]
	F	224 ± 14	<.01	254 ± 15
Triglycerides (mmol/l)	M	1.5 ± 0.6	<.01	2.0 ± 0.4[a]
	F	1.1 ± 0.4	<.05	1.5 ± 0.3
Cholesterol (mmol/l)	M	2.3 ± 0.3[a]	<.01	3.0 ± 0.2
	F	2.6 ± 0.3	<.01	3.2 ± 0.3
Total protein (g/l)	M/F	64 ± 4	NS	63 ± 7
Creatinine (μmol/l)	M/F	43 ± 5	<.01	50 ± 5
Urea (mmol/l)	M/F	6.7 ± 0.6	<.05	7.1 ± 0.9
Ca (mmol/l)	M/F	2.6 ± 0.1	NS	2.5 ± 0.2
P (mmol/l)	M/F	1.8 ± 0.4	<.05	2.2 ± 0.8

[a] Sex differences at 1 percent level.

T-cells. There are 2 alleles named RT6.1 and RT6.2 in the rat. BB rats possess the RT6.1 allele and are unable to express this alloantigen on peripheral lymphocytes. It is assumed that the peripheral T-cell pool of BB rats consists almost exclusively of immature lymphocytes. There are several studies and speculations trying to explain the causes of this phenomenon (1). Some of them suppose that the lymphopenia per se is responsible for the undetectable level of RT6.1 positive cells in the BB rat (16-20). The RT6 gene is located on chromosome 1 and is, therefore of BB/OK origin in the BB.LL rat. The normal level of RT6.1 positive cells in this newly established congenic BB.LL rat strain could support the assumption that the lymphopenia per se causes the undetectable level of RT6.1 positive cells and that the RT6 gene of the BB/OK rat is intact as in diabetes-resistant rat strains. Because cellular and humoral immunity interact the question arises whether the other immunological abnormalities of the BB rat are restored in BB.LL rats. Besides T-lymphopenia BB rats are characterized by antibodies to spleen lymphocytes, gastric parietal cells, smooth muscle cells and thyroid colloid as well as insulin autoantibodies (IAA) and antibodies against pancreatic islet cell surface (ICSA) and cytoplasmatic antigens (ICA) or glutamic acid decarboxylase (GAD) as well as diminished lymphocyte proliferation (1). Whether these abnormalities are also connected with lymphopenia can now be answered with this newly established BB.LL rat strain.

The blood pressure QTL around the *Npy* locus was found to elevate SBP and DBP in a (SHR × WKY) segregating population. In the congenic BB.LL strain where the *Npy* gene and a region of about 1 cM distal and of 10 cM proximal are of SHR origin SBP, DBP and also HR were comparable with those of the recipient strain BB/OK. This finding suggests that the putative QTL around the *Npy* locus seems to represent a false positive linkage to blood pressure as far as the P value for linkage was relatively low in this cross hybrids (P=0.029) (10). Therefore, the requirement of a P value of 5×10^{-5} in order to be ensured that the probability is at most 5% of a QTL occurring by chance, should be fulfilled (21). Moreover, our findings support the necessity to create congenic strains to evaluate the physiologic effect of putative QTLs. Recently we were able to demonstrate that blood pressure QTLs detected in cross hybrids of stroke prone SHR(SP) and normotensive WKY rats contribute to blood pressure increase. Four congenic BB.SHR rat strains clearly indicated by telemetric measurement that each single blood pressure QTL of SHR rats transferred onto the genetic background of BB/OK rats caused a significant increase of SBP (+ 6 to + 17 mmHg) and influenced differently DBP, HR and MA (12). Despite no blood pressure increasing effect was observed in BB.LL rats the significant difference found in MA between BB/OK and BB.LL rats suggest that within this transferred region another than blood pressure affecting QTL must be located. The MA of BB.LL rats was significantly higher in BB.LL than in BB/OK rats and is comparable with those of age-matched SHR males (30.4 ± 2.7 vs. 29.8 ± 5.1 movements/5 min) indicating that within this transferred region a QTL has to be localised influencing MA of SHR and, therefore, also of BB.LL rats. A candidate gene may be *Npy* coding a vasoconstrictor peptide which is widely distributed in the central and peripheral nervous system. The neuropeptide Y is proposed to act as regulator of non-adrenergic sympathic activity, pituitary hormones and renin secretion, appetite and energy (22). In the same region the receptor of another neuropeptide, substance P (*Spr*) is mapped. Several studies have shown that the activation of substance P receptor as well as an increase of substance P concentration elevate the activity in rats (23-25). Therefore, the *Spr* may also be a candidate for high MA in BB.LL rats.

TABLE 4

24h Excretion of Urine Constituents in Male (M) and Female (F) Nondiabetic BB/OK and Congenic BB.LL Rats

No. of males:females	Sex	BB/OK 10:6	P value	BB.LL 11:5
Creatinine clearance (ml/min/100g)	M/F	.51 ± .14	NS	.46 ± .13
Albumin (μg/24 h)	M/F	5 ± 4	NS	4 ± 4
Total protein (mg/24h)	M	21 ± 11[a]	NS	28 ± 17[aa]
	F	13 ± 10	NS	10 ± 2
Urea (mmol/24h)	M/F	8.4 ± 2.1	<.01	10.4 ± 3.0
Na (mmol/24h)	M/F	1.7 ± 0.4	<.01	2.1 ± 0.7
K (mmol/24h)	M	3.8 ± 0.8	<.01	5.0 ± 0.5[aa]
	F	3.4 ± 0.7	<.01	4.4 ± 0.2
Ca (μmol/24 h)	M/F	115 ± 75	NS	136 ± 38
P (μmol/24 h)	M/F	2.3 ± 2.5	<.05	1.3 ± 0.6

Sex differences at 5 ([a]) or 1 ([aa]) percent level.

Interactions between genes of the transferred region and the genetic background of BB/OK rats should be responsible for the new phenotypes in BB.LL rats seen neither in the chromosomal BB/OK recipient nor the SHR/Mol donor strain (12). Considering that the body weight and serum lipids are significantly lower in the chromosomal SHR donor than in the recipient BB/OK strain (12) the obviously increased body weight and serum lipids in BB.LL rats can only be explained by gene interactions. In this case the neuropeptide Y of SHR origin may also be a candidate gene interacting with background genes of BB/OK rats and leading to these new phenotypes. However, to get an answer further studies are necessary not only to evaluate the meaning of the *Npy* for the new phenotypes of BB.LL rats but also to find out possible consequences of the restoration of lymphopenia for the other immunological abnormalities described in the diabetes-prone BB rat.

ACKNOWLEDGMENTS

We thank Marlies Baumann, Silvia Sadewasser, and Karin Titze for expert technical assistance. This work was supported by Grant Kl 771/2-2 of Deutsche Forschungsgemeinschaft.

REFERENCES

1. Crisá, L., Mordes, J. P., and Rossini, A. A. (1992) *Diabetes Metab. Rev.* **8,** 9–37.
2. Jacob, H. J., Pettersson, A., Wilson, D., Mao, Y., Lernmark, A., and Lander, E. S. (1994) *Nature Genet.* **7,** 215, correction.
3. Guttmann R. D., Colle, E., Michel, F., and Seemayer, T. (1983) *J. Immunol.* **130,** 1732–1735.
4. Jackson, R. A., Buse, J. B., Rifai, R., Pelletier, D., Milford, E. L., Carpenteŕ, C. P., Eisenbarth, E. S., and Williams, R. M. (1984) *J. Exp. Med.* **159,** 1629–1636.
5. Klöting, I., and Vogt, L. (1990) *Diabetes Res.* **15,** 37–39.
6. Klöting, I., Vogt, L., and Serikawa, T. (1995) *Diabet. Metab.* **21,** 338–344.
7. Brown, D. M., Steffes, M. W., Thibert, P., Azar, S., and Mauer, S. M. (1983) *Metabolism* **32,** 131–135.
8. Buang, R. D. (1994) *in* Handbook of Hypertension (Ganten, D., and de Jong, W., Eds.), pp 122–123, Elsevier Science, New York.
9. Berg, S., Dunger, A., Klöting, I., and Schmidt, S. (1995) *Autoimmunity* **21,** 7.
10. Katsuya, T., Higaki, J., Zhao, Y., Miki, T., Mikami, H., Serikawa, T., and Ogihara, T. (1993) *Biochem. Biophys. Res. Comm.* **192,** 261–267.
11. Klöting, I., and Vogt, L. (1991) *Diabetes Res.* **18,** 79–87.
12. Klöting, I., Berg, S., Kovacs, P., Voigt, B., Vogt, L., and Schmidt, S. (1997) *Ann. N. Y. Acad. Sci.* **827,** in press.
13. Kuttler, B., Volk, H. D., Kauert, C., Diamantstein, T., and Hahn, H. J. (1990) *Exp. Clin. Endocrinol.* **95,** 64–69.
14. Kuttler, B., Lehmann, M., Lacha, J., Brock, J., and Hahn, H. J. (1994) *Transplant. Proc.* **26,** 728–729.
15. Hedrich, H. J., Fangmann, J., Reetz, I. C., Klöting, I., Kornerup-Hansen, A., and Wonigeit, K. (1990) *Transplant. Proc.* **22,** 2570–2571.
16. Greiner, D. L., Handler, E. S., Nakano, K., Mordes, J. P., and Rossini, A. A. (1986) *J. Immunol.* **136,** 148–151.
17. Lang, F., and Kastner, W. (1989) *Eur. J. Immunol.* **19,** 1785–1789.
18. Thiele, H. G., Koch, F., Haag, F., and Wurst, W. (1989) *Thymus* **14,** 137–143.
19. Colle, E., Fuks, A., Poussier, P., Edouard, P., and Guttmann, R. D. (1992) *Diabetes* **41,** 1617–1623.
20. Crisa, L., Sarkar, P., Waite, D. J., Friedrich, F. H., Koch-Nolte, F., Rajan, T. V., Mordes, J. P., Handler, E. S., Thiele, H. G., and Rossini, A. A. (1993) *Diabetes* **42,** 688–695.
21. Lander, E. S., and Schork, N. J. (1994) *Science* **265,** 2037–2048.
22. Heilig, M., and Widerlov, E. (1990) *Acta Psychiatr. Scand.* **82,** 95–114.
23. Krzhanovskii, G. N., Kucherianu, V. G., Godlevskii, L. S., and Mazarati, A. D. (1992) *Biull. Eksp. Biol. Med.* **113,** 16–19.
24. Mason, G. S., and Elliott, P. J. (1992) *Neuropharmacology* **31,** 757–760.
25. Napier, T. C., Mitrovic, I., Churchill, L., Klitenick, M. A., Lux, X. Y., and Kalivas, P. W. (1995) *Neuroscience* **69,** 59–70.