American Journal of Pathology, Vol. 128, No. 2, August 1987

RAPID COMMUNICATION

A Pancreatic Venular Defect in the BB/Wor Rat

GUIDO MAJNO, MD, ISABELLE JORIS, PhD, EUGENE S. HANDLER, PhD, JAMES DESEMONE, MD, JOHN P. MORDES, MD, and ALDO A. ROSSINI, MD

From the Departments of Pathology and Medicine, University of Massachusetts Medical School, Worcester, Massachusetts

BB rats develop spontaneous autoimmune diabetes mellitus characterized morphologically by insulitis, an inflammatory lymphocytic infiltration of the islets of Langerhans. To investigate the role of the vascular endothelium of the pancreas in this destructive process, the authors injected diabetes-prone (DP) and diabetes-resistant (DR) BB/Wor rats as well as other nondiabetic strains of rats with Monastral blue B, a colloidal pigment that identifies leaky microvasculature. They found evidence of a venular defect limited to the pancreas that is specific to the BB rat. Light- and electron-microscopic evidence suggests that this defect is due to a population of trapped (marginating) intravascular monocytes, which may be activated by the colloidal pigment and release vasoactive mediators. (Am J Pathol 1987, 128:210–215)

A SUBSTANTIAL BODY of evidence now supports the conclusion that human insulin-dependent diabetes mellitus is autoimmune in origin.[1] Additional data suggest that the pathogenesis of diabetes in the spontaneously diabetic BB rat also has an autoimmune basis, and this animal may be regarded as a useful model of the human disorder.[2,3] At least three well-developed lines of evidence are consistent with the autoimmune hypothesis of BB rat diabetes. First, the disease is characterized morphologically by the presence of insulitis, an inflammatory lymphocytic infiltration of the islets of Langerhans.[4] Second, a number of both immunosuppressive and immunomodulatory procedures can either reverse or prevent the disease.[3] Third, BB rat diabetes can be adoptively transferred to naïve recipients by means of mitogen activated splenic lymphocytes from acutely diabetic rats.[5]

Since autoimmune processes like those observed in the BB rat should involve the diapedesis of lymphoid elements from the circulation to the target tissue, possibly together with vascular leakage, it is reasonable to infer that small blood vessels should play a role in the pathogenesis of the disorder. We therefore decided to investigate the role of the pancreatic microvasculature in the BB/Wor rat. Using Monastral blue B (MbB), a colloidal pigment that labels leaky microvasculature, we found evidence of a venular defect limited to the pancreas of the BB rat.

Materials and Methods

Animals

Male and female diabetes-prone BB rats (DP-BB/Wor rats) were obtained from the colony at the University of Massachusetts in Worcester. The cumulative incidence of diabetes in these rats varies from 40% to 70%, and > 85% of all cases appear between 60 and 120 days of age. The frequency of diabetes before 60 days of age is <0.5%. Diabetes-resistant (DR-BB/Wor) rats from the same colony were also used; these were derived from diabetes-prone forebears, but have been bred for resistance to the disease.[6] Splenic and peripheral lymphocyte numbers and subset percentages in DR-BB/Wor rats are similar to those of normal Wistar Furth rats.[7] At the time of these experi-

Supported in part by Grants HL25973, HL33529, and DK25306 from the National Institutes of Health.

Accepted for publication June 5, 1987.

Address reprint requests to Guido Majno, MD, Department of Pathology University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01605.

ments, DR-BB/Wor rats had been inbred for >26 generations, and fewer than 1.0% (N >8,000) had become diabetic. New England Deaconess Hospital (NEDH) rats obtained from a colony maintained in our laboratory and Wistar Furth (WF) and Lewis (LEW) rats obtained from the National Cancer Institute, Frederick, Maryland, were used as controls.

All animals were maintained in accordance with recommendations in the *Guide for the Care and Use of Laboratory Animals* (Department of Health, Education, and Welfare Publication NIH 78-23, 1985) and the guidelines of the Animal Care Advisory Committee of the University of Massachusetts School.

Test of Microvascular Function

The integrity of the microvascular endothelium was determined by means of the technique of *vascular labeling*,[8] which identifies leaky vessels in the microcirculation. In brief, a colloidal suspension of a pigment such as MbB (copper phthalocyanine), with a particle size of up to 500 Å, is injected intravenously.[9] Wherever gaps occur in the endothelium, plasma escapes and filters through the basement membrane, which remains intact. Particles of colloidal pigment suspended in the plasma are retained by the basement membrane and form deposits that label the abnormally permeable vessel. These deposits are easily visible by light and electron microscopy. In these experiments MbB (Lot #65-F-6132, Sigma Chemical Co., St. Louis, Mo) was injected into the tail vein of recipient rats at a dose of 0.2 ml/100 g body weight. To maximize labeling of leaky vessels, MbB was injected three times, either on a single day at 2-hour intervals or on successive days, or three times over 6 days. Similar results were obtained with all procedures. All animals were sacrificed 2–24 hours after the last injection, which allowed ample time for the circulating pigment to be cleared by the reticuloendothelial system.

Experimental Protocols

We tested four groups of BB and non-BB rats. These included 42 nondiabetic DP-BB/Wor ranging in age from 20 to 200 days, 20 DR-BB/Wor rats 22 to >80 days of age, and 23 acutely or chronically diabetic DP-BB/Wor rats. As nondiabetic controls we tested 15 WF, 5 LEW, and 3 NEDH rats. Fifteen additional controls (5 WF, 5 LEW, 5 NEDH) were also tested; but the pancreas, instead of being examined histologically, was scanned by the more rapid method of transillumination, to be described further.

All animals were sacrificed in an atmosphere of 100% CO_2, as is customary in this laboratory. At autopsy the pancreas was inspected grossly, fixed flat in 10% neutral formalin, and embedded in paraffin. Histologic sections were cut parallel to the flat surface. Samples were also taken from the organs of the neck (trachea, esophagus, thyroid, striated muscle) as well as from salivary glands, heart, lungs, liver, and spleen. Histologic sections were stained with hematoxylin and eosin or with eosin only to facilitate identification of MbB deposits. Samples for electron microscopy were prepared by flooding the pancreas *in situ* with 3% glutaraldehyde in cacodylate buffer, followed by rapid trimming into 1-cu mm cubes, and embedding in Epon.

In a second series of experiments we used a simplified method devised for surveying MbB labeling in the entire pancreas. The pancreas was excised and placed between two 50 × 75-mm glass microscope slides. Using surgical gauze forceps, it was gently but firmly compressed between the slides and dipped in boiling water for 45 seconds. It was then allowed to cool and partially air-dry on one of the slides. Thereafter, it was cleared in two baths of glycerin, each one lasting 12–24 hours, and permanently mounted in glycerin jelly.[8] These "squash preparations" were studied microscopically by transillumination.

Results

Immediately after the intravenous injection of MbB, the snout, ears, and paws of all rats became bluish, but returned to normal within 1 hour. At autopsy, the liver, spleen, and, to a lesser extent, the adrenals of all rats were dark blue. The pancreases of all control (non-BB/Wor) rats were of a normal pale yellow or pink color, occasionally studded with a deep blue lymph node. The same was true for about 75% of the DP and DR-BB/Wor rats; in the remaining, the pancreas was bluish, and in 4 cases definitely blue; the two extremes are shown in Figure 1.

Under the dissecting microscope the blue-stained pancreases showed a faint blue branching pattern corresponding to the distribution of the venules, better seen in the squash preparations (Figure 2). MbB-labeled venules were found in pancreases from 57% of the DP rats, 40% of the DR-BB/Wor rats, and 65% of diabetic rats (Table 1), but in none of the 38 non-BB rat controls. No blue deposits were observed grossly in any other organ, with two exceptions; in 8 rats (6 DP-BB/Wor, 1 DR-BB/Wor, 1 WF) the trachea was sky blue, and in 2 DR rats the thyroid was bluish.

Microscopically, MbB deposits were observed, as expected, in the liver, spleen, milky spots of the omentum, and pancreatic lymph nodes. The blue tra-

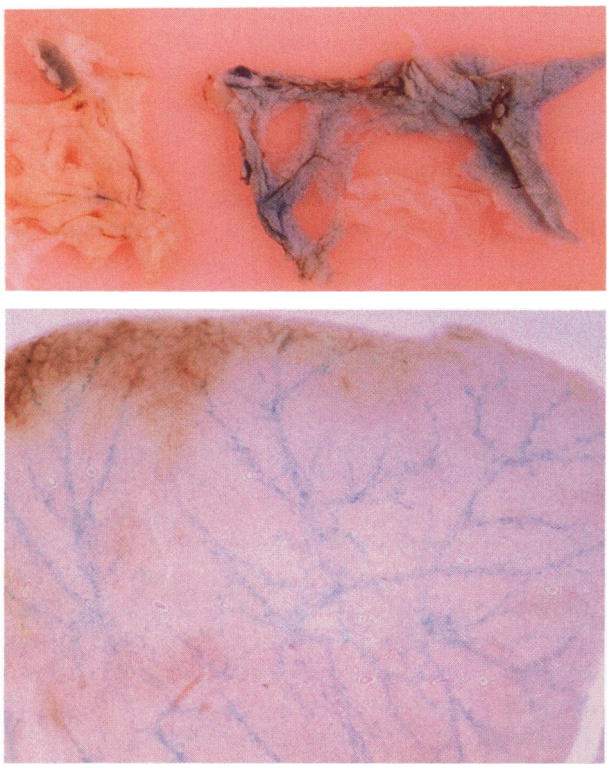

Figure 1—Gross appearance of the pancreas in diabetes-prone BB/Wor rats after intravenous injection of Monastral blue B, illustrating two possible extreme effects: *left*, no blue deposition (except in pancreatic lymph node); *right*, intense blue deposition. (× 1.25) **Figure 2**—Squash preparation of the pancreas of a diabetes-prone BB/Wor rat after intravenous injection of Monastral blue B. The blue arborizations correspond to labeled venules. (Unstained, × 28)

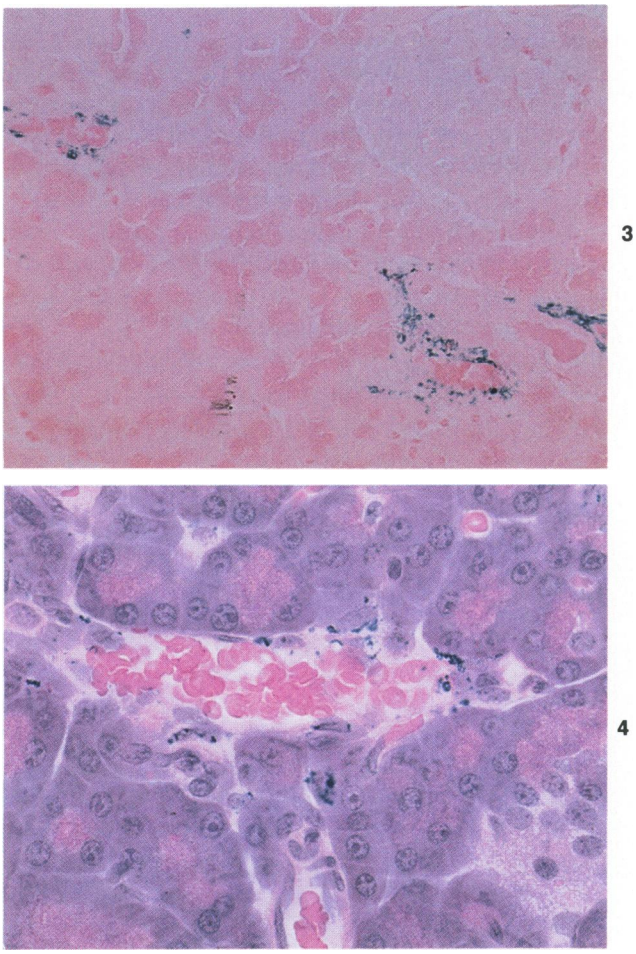

Figure 3—Histologic section of the pancreas of a diabetes-prone BB/Wor rat: venular and perivenular deposits of pigment; no deposit in the islet. (Eosin only, × 160) **Figure 4**—Histologic section of the pancreas of a diabetes-resistant BB/Wor rat: deposits of Monastral blue B in the wall of a venule; no deposit related to the nearby islet. (H&E, × 440)

cheas had an acutely inflamed mucosa (acute tracheitis) clearly irrelevant to the experiment. The bluish thyroids showed chronic thyroiditis, as previously observed.[10] The pigment was present in macrophages scattered throughout the thyroid. No MbB was found in other organs, except for occasional minuscule deposits. The pancreases that had appeared grossly blue showed MbB in the walls of many venules (Figures 3 and 4), but labeled venules were present also in pancreases of DP and DR rats that appeared grossly normal. Sometimes they were diffusely present throughout the organ; other times only two or three were seen in a given section. At high power the blue deposits could be located either between the endothelial cells and the basement membranes or in vacuoles contained within the endothelial cells and/or pericytes. Monocytes containing MbB were found marginating in venules labeled or unlabeled. Electron microscopy confirmed that labeled venules contained MbB trapped against the basement membrane, as well as in phagosomes within the endothelium, in pericytes and in marginating macrophages (Figure 5). Many of these showed signs of activation. One venule showed a gap next to an MbB-labeled endothelial cell. The presence of labeled venules in the DP rats did not depend on the presence of insulitis. Some insulitis was seen in 7 of the DP rats (Table 1), but the labeled venules were outside the inflammatory foci, and no pigment was present in the inflamed islets. No edema was found, as might have been expected in the neighborhood of chronically leaky venules.

Table 1—Findings in the Three Groups of BB/Wor Rats

	Total number of rats	Number with leaky venules	Number without leaky venules	Percent with labeled venules
Diabetes-prone	42 (7)	24 (5)	18 (2)	57
Diabetic	23 (23)	15 (15)	8 (8)	65
Diabetes-resistant	20 (4)	8 (4)	12 (0)	40

Figures in parenthesis refer to the number of animals with insulitis and related changes.

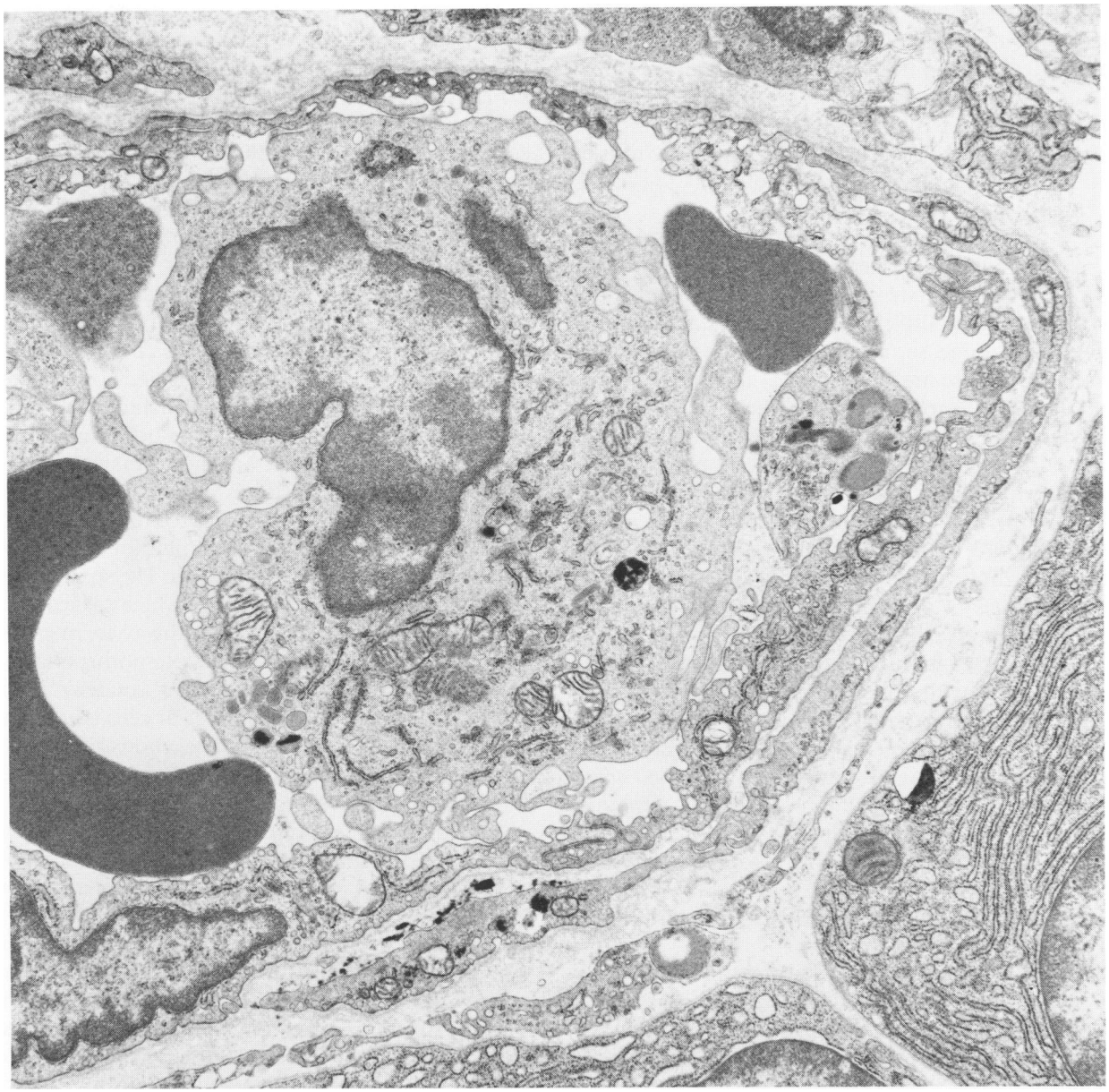

Figure 5—Electron micrograph from the pancreas of a diabetes-prone BB/Wor rat after intravenous injection of Monastral blue B. A monocyte is trapped in the venule; its ruffled membrane is consistent with activation. Dark particles of pigment are seen in the monocyte phagosomes and in the wall of the adjacent venule. (Lead citrate, × 10,300)

The squash preparations of pancreas confirmed the results obtained with histology. The characteristic branching arrangement of MbB-labeled venules was especially obvious (Figure 2).

Discussion

The data presented here suggest that a venular defect is present in BB/Wor rats and that this defect is specific to the pancreas. The defect, furthermore, is present not only in diabetic and diabetes-prone rats but in diabetes-resistant BB/Wor rats as well; it was never found in 38 rats of three inbred strains. It is not related to age (between 20 and 200 days) and does not correlate with the diabetic condition *per se*, although we suspect that a link does exist in a manner yet to be understood.

Histologic sections show that the pigment is intramural and not incorporated within thrombi (Figures 3 and 4); electron microscopy proves that some pigment deposits are trapped against the basement membrane (Figure 5). By definition, this corresponds to the phenomenon of vascular labeling[8] and implies that endothelial gaps must have existed while the col-

loidal material was circulating in the plasma. We should point out that these findings are fundamentally different from those of others who described deposits of MbB[11] or Evans blue[12] in the islets of rats given the beta cytotoxic agent streptozotocin; those deposits indicate vascular damage secondary to islet cell necrosis, conditions that do not apply to our experiments.

The labeling is strictly limited to the venules; no labeled capillaries or arterioles were found. This specificity may relate to the fact that venules are the primary target of permeability-increasing agents such as histamine, serotonin, bradykinin,[8] and leukotriene E_4.[13] These mediators appear to induce venular endothelial cells to contract and thus to create intercellular gaps. Venules are also a common target of pathologic processes such as leukocyte margination, immune responses, and microscopic hemorrhages. It is not surprising that the permeability defect in our model should be present in the exocrine pancreas, since no venules are present in the pancreatic islets[14]; small venules begin to form only as the capillaries emerge from the islets.

All the observations discussed above indicate that we are dealing with a phenomenon of endothelial leakiness analogous to that induced by mediators of acute inflammation.[8] This analogy, however, brings us face-to-face with a puzzling fact: *there is no acute inflammation.* While foci of chronic inflammation (insulitis) do occur, they do not correlate with the venular defect. In most DP-BB/Wor and DR-BB/Wor rats, insulitis is absent. Could there be venular leakage without inflammation? No such condition is known to us.

We see only two ways to interpret the facts. *One is to assume that there is indeed, in some BB rats, a persistent venular defect* that leads to a type of leakage heretofore unknown. This is not impossible, but highly unlikely for several reasons. Persistent vascular leakage should lead to edema of the pancreas; no such edema is apparent. To this one might object that a very low grade venular leakage might not necessarily produce edema (lymphatic drainage might have adapted to the situation). However, a venular defect that allows significant amounts of pigment to escape during a relatively short time (on the order of 30–60 minutes) does not seem compatible with the notion of a minimal escape of fluid. Last, if the pigment can escape, plasma lipoproteins and chylomicrons should also be able to escape, leading to extensive phagocytosis by pericytes and perivascular macrophages; no such activity is visible. We must conclude that the hypothesis of a persistent venular leakage hangs on a thin thread.

The alternative hypothesis is that in the pancreas of the BB rat there is no constant venular leakage, but only a predisposition to venular leakage, and that *the actual leakage is brought about in susceptible animals by the injection of Monastral blue B.* How could this colloidal material induce the defect? Two cell targets are accessible to circulating colloidal particles: leukocytes and endothelium. Much evidence points to the former, ie, to the *activation of intravascular monocytes.*

In the pancreas of our BB rats, many venules (including labeled venules) harbor marginating monocytes; and most of these monocytes have obviously phagocytized MbB (Figure 5). Phagocytosis is concomitant with activation, which includes the secretion of vasoactive mediators.[15,16] Furthermore, in a recent study on sheep,[17] it was shown that an intravenous injection of MbB causes vascular and bronchial spasm in the lung; this was attributed to the activation of the resident population of pulmonary macrophages characteristic of this species. The spasm could be prevented with indomethacin, which suggests that products of the cyclooxygenase pathway are involved. This observation suggests a highly tempting analogy: in one case (the sheep) a physiologic, "resident" population of intravascular mononuclear cells is activated to the point of inducing adverse reactions; in the other case (the BB rat) a pathologic population of "trapped" intravascular monocytes could be similarly activated.

Activation of the endothelium must also be considered; indeed it may be complementary. If the monocytes are trapped, this could be the effect of a previous activation of the endothelium; recent studies have shown that cultured endothelium activated by IL-1 becomes more sticky for leukocytes.[18] We have no evidence that the endothelium, let alone the endothelium of the pancreas specifically, could be activated by circulating MbB. It could conceivably be activated by an antiendothelial antibody; various autoantibodies are prevalent in the BB rat.[19] Certainly, human pancreatic endothelium can be activated (to become HLA-DR-positive) in established insulitis.[20]

We therefore propose the following working hypothesis. The pancreas of the BB rat contains a population of trapped intravascular monocytes (as a result of a yet unknown endothelial abnormality). These monocytes, if activated, can induce venular leakage. Studies are in progress to verify the various steps of this hypothesis; however, we believe that the riddle of venular labeling in the BB rat is no longer as impenetrable as it seemed a few months ago.[21] It is also intriguing *per se*, because the occurrence of venular leakage in the absence of inflammation has, as far as we know, no precedent in the literature.

References

1. Cahill GF Jr, McDevitt HO: Insulin-dependent diabetes mellitus: The initial lesion. N Engl J Med 1981, 304:1454–1465
2. Marliss EB, Nakhooda AF, Poussier P, Sima AAF: The diabetic syndrome of the "BB" Wistar rat: Possible relevance to Type 1 (insulin-dependent) diabetes in man. Diabetologia 1982, 22:225–232
3. Rossini AA, Mordes JP, Like AA: Immunology of insulin-dependent diabetes mellitus. Ann Rev Immunol 1985, 3:291–322
4. Nakhooda AF, Like AA, Chappel CI, Murray FT, Marliss EB: The spontaneously diabetic Wistar rat: Metabolic and morphologic studies. Diabetes 1977, 26:100–112
5. Koevary S, Rossini AA, Stoller W, Chick W, Williams RM: Passive transfer of diabetes in the BB/W rat. Science 1983, 220:727–728
6. Like AA, Rossini AA: Spontaneous autoimmune diabetes mellitus in the BioBreeding/Worcester rat. Surv Synth Path Res 1984, 3:131–138
7. Greiner DL, Handler ES, Nakano K, Mordes JP, Rossini AA: Absence of the RT-6 T cell subset in diabetes-prone BB/W rats. J Immunol 1986, 136:148–151
8. Majno G, Palade GE, Schoefl GI: Studies on inflammation: II. The site of action of histamine and serotonin along the vascular tree: A topographic study. J Biophys Biochem Cytol 1961, 11:607–626
9. Joris I, DeGirolami U, Wortham K, Majno G: Vascular labelling with Monastral blue B. Stain Technol 1982, 57:177–183
10. Rossini AA, Faustman D, Woda BA, Like AA, Szymanski I, Mordes JP: Lymphocyte transfusions prevent diabetes in the Bio-Breeding/Worcester rat. J Clin Invest 1984, 74:39–46
11. Sandler S, Jansson L: Vascular permeability of pancreatic islets after administration of streptozotocin. Virchows Arch [Pathol Anat] 1985, 407:359–367
12. Beppu H, Maruta K, Kürner T, Kolb H: Diabetogenic action of streptozotocin: essential role of membrane permeability. Acta Endocrinol (Copenh) 1987, 114:90–95
13. Joris I, Majno G, Corey EJ, Lewis RA: The mechanism of vascular leakage induced by leukotriene E_4: Endothelial contraction. Am J Pathol 1987, 126:19–24
14. Bonner-Weir S, Orci L: New perspectives on the microvasculature of the islets of Langerhans in the rat. Diabetes 1982, 31:883–889
15. Adams DO, Hamilton TA: The cell biology of macrophage activation. Ann Rev Immunol 1984, 2:283–318
16. Hartung H-P, Kladetzky RG, Melnik B, Hennerici M: Stimulation of the scavenger receptor on monocytes-macrophages evokes release of arachidonic acid metabolites and reduced oxygen species. Lab Invest 1986, 55:209–216
17. Albertine KH, Staub NC: Vascular tracers alter hemodynamics and airway pressure in anesthetized sheep. Microvasc Res 1986, 32:279–288
18. Bevilacqua MP, Pober JS, Wheeler ME, Cotran RS, Gimbrone MA: Interleukin-1 activation of vascular endothelium: Effects on procoagulant activity and leukocyte adhesion. Am J Pathol 1985, 121:393–403
19. Like AA, Appel MC, Rossini AA: Autoantibodies in the BB/W rat. Diabetes 1982, 31:816–820
20. Bottazzo GF, Dean BM, McNally JM, MacKay EH, Swift PGF, Gamble DR: In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis. N Engl J Med 1985, 313:353–360
21. Majno G, Joris I, Handler ES, Mordes JP, Rossini AA: A pancreatic venular defect in the BB/W rat (Abstr). Diabetologia 1986, 29:567A

Acknowledgments

We thank Olita Treimanis, Eva Moring, Jean M. Underwood, and Linda Paquin for technical help, Christopher D. Hebert for the photographic prints, and Jane M. Manzi and Barbara Papazian for preparation of the manuscript.