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Prevention of Diabetes in Rats by Bone Marrow Transplantation

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Hyperglycemia, hypoinsulinemia and ketonemia often develop abruptly in previously normal young "BB" rats. The syndrome mimics human juvenile diabetes closely and is, thus, appropriate for assessing pancreatic transplantation. Transplantation of islet cells from closely histocompatible Wistar Furth (WF) donors resulted in permanent normoglycemia when immunosuppression with ALS was given. However, when islet cells from nondiabetic "BB" donors were transplanted to *non-immunosuppressed* diabetic "BB" recipients, only transient normoglycemia followed. Transplantation of WF islet cells also failed in diabetic "BB" rats which were tolerant of WF antigens, again suggesting destruction of transplanted islet cells by the original disease process—possibly autoimmunity. Evidence for autoimmunity was strengthened by the finding that newly diabetic "BB" rats could be rendered normoglycemic by immunosuppression. Since genetic susceptibility to spontaneous autoimmune diabetes is unique to some members of the "BB" stock, an attempt was made to alter their vulnerability by modifying their cellular immune system. Accordingly, 50 million bone marrow cells from WF donors were inoculated into half the newborn members of "BB" litters, leaving the littermates as unmodified controls. Most bone marrow recipients were protected, only four of 37 (10.8%) ever becoming diabetic, while the incidence of diabetes in noninoculated littermates was 22 of 39 (56.4%). The ultimate goal in human diabetes, which also seems very likely to be an autoimmune disease, may not be replacement of destroyed islet cells but identification of potentially susceptible children and prevention of islet destruction by immunologic manipulation.

DIABETES MELLITUS a disease recognized by the ancient Egyptians, is assuming an increasingly important position in modern health care. Prior to the advent of insulin therapy, in 1922, the usual outcome of the severe juvenile onset type of diabetes was prompt death from ketoacidosis[1]. Insulin now prevents early deaths from acute metabolic complications, and prolongs the life of diabetics. However, it also allows them to survive long enough for the chronic complications to become manifest, with the result that diabetes is now the second commonest cause of blindness, the third most common cause of renal failure and the fifth commonest cause of death in the United States, where the annual cost of the disease is estimated to be five to six billion dollars[2]. Furthermore, lengthening the life of diabetics into the reproductive years has been associated with a progressive increase in the incidence of this inheritable condition with the result that 20% of children being born today can be expected to develop some form of the disease.

Unfortunately, relatively little progress has been made in discovering the cause of diabetes since Banting and Best demonstrated the effectiveness of administering pancreatic extracts in palliation of the metabolic complications. Treatment of ketoacidosis has now been perfected to the extent that death is rare in the early stages of diabetes. Thus, pancreatic tissue from recently afflicted individuals rarely becomes available for pathologic or microbiologic studies which might help elucidate the cause. Furthermore, experimental approaches to the study of diabetes have been seriously hindered by the lack of appropriate animal models. Hyperglycemic states artificially induced by pancreatectomy or by chemicals such as alloxan or streptozotocin are relatively useless for etiologic studies of spontaneous diabetes. Although several naturally occurring diabetic states have been found in mice and rats, none of these are marked by insulin deficiency or susceptibility to ketoacidosis[3]. Instead, they are characterized by obesity and insulin resistance and resemble mild adult onset diabetes rather than the insulin dependent juvenile form. Indeed, until recently, the Chinese hamster was the only model of spontaneous diabetes in small mammals in which insulin deficiency was a part of the syndrome[4].

However, a newly described type of spontaneous diabetes occurring in rats promises to be the animal model which most closely resembles human insulin-dependent diabetes. In 1974, spontaneous hypergly-

Presented at the Annual Meeting of the American Surgical Association, Chicago, Illinois, April 22–24, 1981.

Supported in part by USPHS grants AM26007, AM07314, CA15822, The Reynolds Foundation, and the Thomas McCabe Fund.

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cemia was recognized in several members of a colony of outbred rats in a commercial breeding colony (Bio Breeding Laboratories, Ottawa, thus "BB" rats), a highly unusual finding since this species is not known to be susceptible to diabetes[5]. Study of additional animals from this stock showed that the disease affected about 2% of the colony. Its onset was abrupt, occurring usually in animals 60–90 days old which had previously been normoglycemic. Unlike other rodent models of diabetes, the syndrome did not include obesity, but was marked by severe hypoinsulinemia and hyperglycemia (250–700 mg/dl). Unless insulin therapy was instituted promptly, most diabetic rats died from ketoacidosis. Another important similarity to human juvenile diabetes was the histologic finding in newly diabetic rats of mononuclear infiltration of the pancreatic islets (insulitis). Since this disease appears to be a close counterpart of human insulin-dependent diabetes, and occurs in a species lending itself to genetic, immunologic and transplantation experiments, it seems inevitable that valuable information will ensue from careful study of this unique syndrome. The present report concerns our preliminary attempts to define etiologic factors in this new animal model and, in so doing, to suggest possible therapeutic measures which might be logical approaches to modifying or preventing diabetes in man.

Materials and Methods

Animals

A breeding nucleus of 19 rats, members of two litters of "BB" rats, was obtained from Bio Breeding Laboratories of Canada Ltd, Ottawa, Ontario, Canada in October, 1977 and another of 48 rats, members of 6 litters from Sir Frederick Banting Research Center, Ottawa in July, 1980. Random breeding of these animals in our animal colony over a three and half year period has yielded more than 250 additional litters, a total of over 2500 rats. To screen young "BB" rats for the onset of diabetes, tail vein blood is obtained from unfasted animals over 60 days of age and plasma glucose levels determined at approximately weekly intervals. Following the onset of diabetes, which we define as a plasma glucose greater than 200 (mg/dl) (although it rarely remains less than 350 (mg/dl) without treatment), the animals received 2–3 units of PZI insulin daily. A mild form of the disease which occurs in a small percentage of "BB" rats can be diagnosed only by recognition of minimal insulitis. Since many of our studies were based on lifetime observation of animals for the appearance of overt diabetes, we did not routinely kill rats for histologic study of the pancreas. Thus, our definition of diabetes, as is the case in man, is based on hyperglycemia.

In addition to the outbred "BB" rats, the following isogenic rat strains, bred and maintained in our own colony, were used: Lewis (L), ACI, BN, and Wistar Furth (WF).

Histocompatibility Typing

The major histocompatibility complex (MHC) genotype was defined by serologic typing and mixed lymphocyte reactions. Reactivities of a panel of rat alloantisera with known specifities for Class I MHC antigens in the rat were studied in a direct complement-dependent cytotoxicity assay, according to the technique described by Götze[6]. Rabbit serum, selected for low toxicity on rat lymphocytes, was the source of complement, and peripheral blood lymphocytes separated by discontinuous Percoll gradients served as target cells.

Class II MHC antigens were studied by unidirectional mixed lymphocyte culture. Thoracic duct lymphocytes (responder cells) from representative inbred rat strains were cocultured with irradiated (1700 R) stimulator lymph node (LN) cells of "BB" rats.

Detection of Islet Cell Antibody (ICA)

Presence of ICA was investigated by means of standard indirect immunofluorescence techniques[7]. Sera collected from acutely diabetic "BB" rats were incubated with thin (5 μ) frozen sections of fresh "BB" or WF rat pancreatic tissue. The sections were then washed, incubated with fluorescein isothiocyanate conjugated rabbit antirat IgG (Cappell Laboratories, Cochranville, PA), and examined by fluorescent microscopy.

Mixed Lymphocyte Culture (MLC)

Analytical MLC were performed by the method of Wilson[8]. Responder lymphocytes, 6×10^5, (obtained by thoracic duct cannulation) were cultured in triplicate with 3×10^5 irradiated stimulator lymphocytes (obtained from lymph nodes) in sterile round bottom microtiter plates. After four days of culture, 0.25 μCi of ^{3}HT-dr (6.7 Ci/mM) diluted in RPMI was added to each well. Twelve to 14 hours later, cultures were harvested onto glass filters. The filters were air dried and then counted in 1 ml aliquots of Econofluor®.

Transplantation of Bone Marrow

Newborn rats (less than 24 hours old) were intravenously inoculated via the orbital branch of the anterior facial vein with 50×10^6 bone marrow cells in 0.2 ml Hanks' solution. The immunologically tolerant state

which is known to be the usual result of this treatment was confirmed by a donor strain skin allograft performed when the rat was 4 weeks of age[9]. Recipients which retained healthy skin allografts for over 200 days were considered tolerant.

Pancreatic Islet Cell Isolation and Transplantation

Islet cells were isolated, by a previously described technique, using collagenase digestion and centrifugation through Ficoll gradients[10]. Transplantation was performed by portal vein inoculation.

Transfer of Autoimmune Insulitis

Transfer experiments involved intravenous or intraperitoneal inoculation of sublethally irradiated (650R) nondiabetic rats with 50–100 × 10^6 thoracic duct lymphocytes from acutely diabetic "BB" rats.

Virus Isolation Procedure

Monolayers of MRC-5, Vero, WIRL and "BB" - embryo cells were directly inoculated with cell free homogenates of heart, liver, spleen, kidney, pancreas and brain tissue obtained from pre-diabetic or acutely diabetic "BB" rats. To investigate the presence of a latent virus in "BB" rat tissues, their confluent cultures were examined with explant cell culture technique and examined for cytopathology.

Results

Several etiologic possibilities of diabetes in the "BB" rat have been explored experimentally; 1) genetic predisposition; 2) viral infection; 3) autoimmunity; 4) immune deficiency. These will be considered separately along with the result of therapeutic maneuvers suggested by each possible cause.

Evidence for Genetic Predisposition

Breeding. The importance of genetic background in susceptibility to diabetes is evidenced by the fact that selective breeding of diabetic or nondiabetic "BB" rats and their descendents either increases or decreases the frequency of the disease. By breeding nondiabetic "BB" rats for several generations a nondiabetic line has been developed in which there is a less than 1% incidence of the disease. On the other hand, the incidence of the disease in our "diabetes-prone line" is currently 58% when both parents are diabetic, and 27% when an affected "BB" rat is bred to a nondiabetic sibling.

Definition of MHC (RT1) genotype. Twenty-two "BB" rats, some of which were diabetic, were typed both serologically and by the MLC reaction for their MHC. All proved to be homozygous for $RT1^u$. To determine if homozygosity at this locus was in any way related to the susceptibility to the disease, the MHC genotypes of two of 30 ("BB" × ACI) F_1 × "BB" backcross rats which developed the disease and two of 40 ("BB" × BN) F_1 × "BB" backcross animals which became diabetic, were determined; all proved to be homozygous for $RT1^u$. It, thus, appears that although the diabetic condition in these rats is not under simple genetic control, the MHC may play a role in its cause.

Virus Infection

Direct inoculation of MRC-5, Vero, WIRL and "BB" embryo cell lines with free homogenates of tissues from over 20 "BB" rats either acutely diabetic, or from young rats presumed to be at high risk of becoming diabetic, failed to show viral replication or cytopathologic changes in any instance. Likewise, attempts to isolate virus from "BB" tissues by explant cell culture were unsuccessful.

Evidence of Autoimmunity

The insulitis lesion. In 22 acutely diabetic "BB" rats, the pancreas was examined histologically within seven days of the onset of hyperglycemia. In all instances, an intense mononuclear infiltration of the islets was seen with small lymphocytes being the predominant cell type in the infiltrate. Eight of these rats had previously undergone pancreatic biopsy prior to 40 days of age, at which time, the pancreatic islets appeared normal in all. The usual correlation of the onset of hyperglycyemia, with an islet lesion morphologically resembling that of allograft rejection, remains one of the strongest pieces of evidence that occurrence of diabetes in "BB" rats has an immune basis.

Islet cell antibodies. In five acutely diabetic rats, the immunofluorescence technique was used to search for IgG in the pancreatic islets with negative results in all. In three other instances, frozen sections of pancreas from normal rats were treated with serum obtained from acutely diabetic rats, and then with an anti-rat IgG conjugated with fluorescein, in an attempt to demonstrate islet cell antibody by this indirect technique. These results were also negative.

Serum from acutely diabetic rats was administered to three normoglycemic "BB" rats (intravenous administration of 1 cc daily for five days) in an attempt to induce diabetes on the basis of serologic factors. All three rats remained normoglycemic. In three other rats, serum from acute diabetics was used in an attempt to damage transplanted isolated islet cells, since these may be more vulnerable to antibody than islets in the

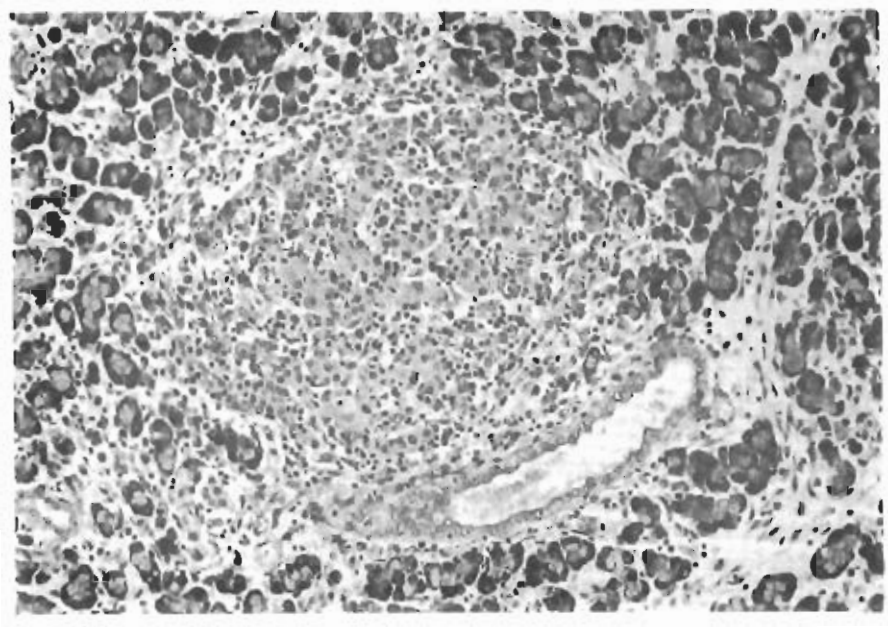

FIG. 1. Diffuse lymphocytic infiltration of the islets such as the one shown here was found in the pancreas of this nondiabetes prone "BB" rat 16 days after inoculation with 100 million thoracic duct lymphocytes from an acutely diabetic "BB" rat (H & E ×160).

intact pancreas [11]. Nondiabetic "BB" rats which had been rendered hyperglycemic with streptozotocin were transplanted with islet cells from nondiabetic "BB" donors, and when normoglycemia had been established for two weeks, the rats were treated with 5 ml of serum (intravenous administration of 1 cc daily) taken from acutely diabetic "BB" rats. These rats also remained normoglycemic.

Passage of diabetes by lymphoid cells. One-half of the littermates from each of two "BB" litters of the nondiabetes-prone strain (a total of six rats) received 50–100 × 10^6 thoracic duct lymphocytes from an acutely diabetic "BB" rat. Sublethal irradiation (650r) was administered to prevent rejection of the inoculated cells. Plasma glucose determinations performed at two day intervals remained normal over a 16 day observation period. The histologic picture of pancreatic biopsies 6 days after cell transfer also were normal. At 16 days, the animals were killed and the pancreas found to be normal in four of the rats. However, in two recipients of diabetic cells, definite insulitis was noticed (Fig. 1). These results, though not conclusive, suggest that some features of the syndrome may be transmitted via "immune" lymphocytes.

Immunosuppression. The possibility that immunosuppression might abort an autoimmune process, and allow healing of the insulitis lesion, was initially suggested by our finding in a previous study that two of 12 "BB" rats treated successfully for diabetes by allogeneic islets and immunosuppression with ALS, when killed after several months, proved to have not only healthy transplanted islet cells, but also normal islet cells in their native pancreas[12]. Like et al. also reported that treatment with radiation or ALS alone was capable of preventing the onset of diabetes in young "BB" rats and sometimes reversed hyperglycemia[13]. We have now treated 15 diabetic "BB" rats with immunosuppression alone. Administration of ALS (obtained from Microbiological Associates, Walkersville, MD.) (1 cc daily) was initiated within seven days of the recognition of hyperglycemia. In six animals, hyperglycemia continued unabated, but nine other rats became normoglycemic within 21 days. When ALS therapy was discontinued after 30 days, four rats remained normoglycemic until death 9–11 months later, and two others are currently normoglycemic two months after ALS was stopped. But, in the remaining three animals, recurrence of diabetes was noticed after one, three, and three months. Histologic examination of the pancreas in rats with recurrent diabetes revealed typical insulitis. Both regression of diabetes in ALS-treated animals, and its recurrence after discontinuation of immunosuppression, argue for the etiologic importance of immunity.

Islet transplantation. Our earliest experiments had shown that islet transplantation was successful in immunosuppressed "BB" diabetics[12]. But since immunosuppression itself was now known to alter the course of the disease, it became necessary to determine the outcome of transplantation in *nonimmunosuppressed* rats to evaluate the possible effect of unmodified autoimmunity on newly transplanted islets. It was anticipated that the interpretation of such transplant studies might be complicated, since syngeneic transplants could not be done in the nonisogenic "BB" rats, and since rejection would be likely to damage alloge-

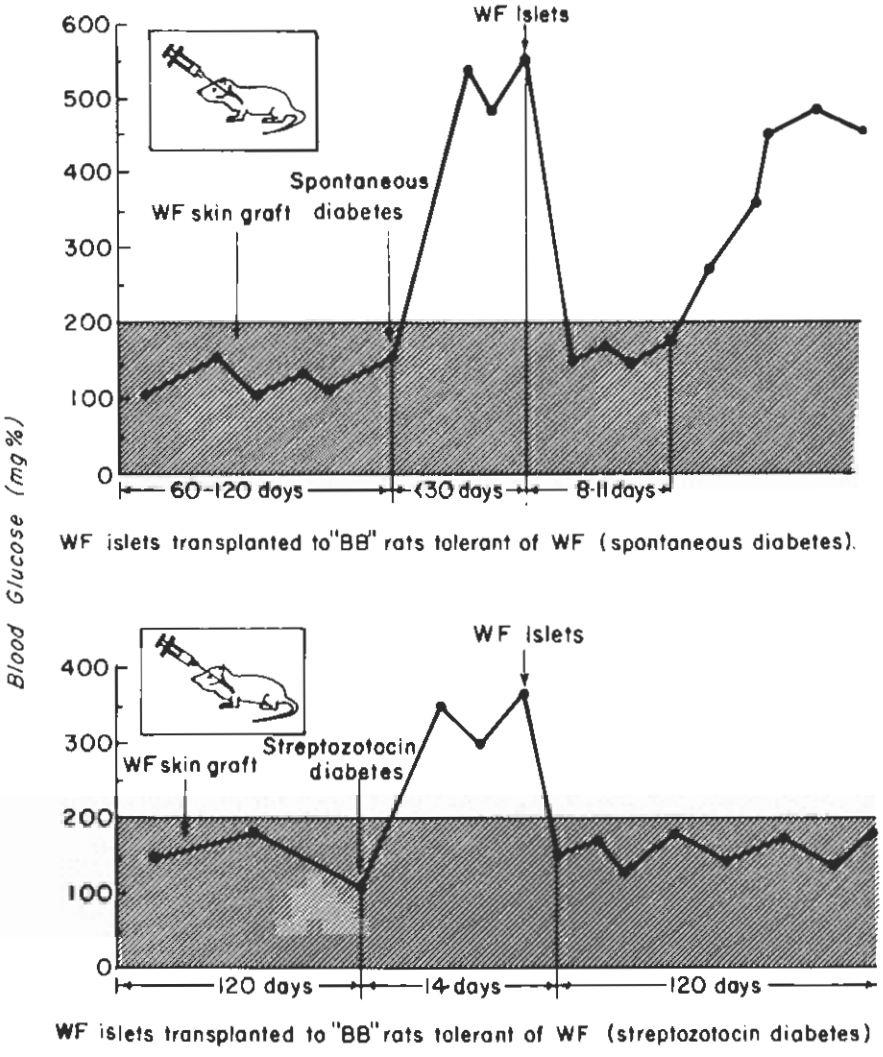

FIG. 2. Two groups of "BB" tolerant of WF rats received transplanted islets from WF donors. In the first group which had hyperglycemia artificially induced by streptozotocin, islet transplantation was permanently successful. In the second group which has spontaneous diabetes the transplanted islets failed rapidly, probably on the basis of autoimmune destruction.

neic islet cells in nonimmunosuppressed recipients. Fortunately, however, members of our closed colony of "BB" rats were quite histocompatible, as evidenced by their MHC identity and by acceptance of skin allografts exchanged within the "BB" stock for more than 100 days in 11 of 16 instances. Thus, it was reasoned that in some instances allografts of islet cells exchanged between "BB" rats might similarly avoid rejection, at least for a considerable period of time. Islet cells from eight different nomoglycemic "BB" donors were used for each allograft to increase the chance that some of the transplanted islet cells would be completely or closely compatible with the recipient. Initially, islet cell transplantation was done in artificially hyperglycemic rats of the "BB" stock. Recipients were selected from a group of "BB" rats which had reached over 150 days of age without developing diabetes, thus becoming unlikely candidates for the spontaneous disease (and presumably for an autoimmune process which might damage islets). These nondiabetic recipients were rendered hyperglycemic (<300 mg/dl) with streptozotocin (65 mg/kg). Each of ten such rats was transplated with islet cells from nondiabetic "BB" donors with the result that normoglycemia ensued in all. In four instances, islet failure occurred and hyperglycemia recurred (apparently from rejection) after 12, 13, 14, and 17 days. However, in the other six recipients, prolonged normoglycemia (60–300 days) was the result.

An identical islet transplantation protocol was then used in eight *spontaneously* diabetic "BB" recipients whose hyperglycemia was of recent onset (<30 days) so that a putative autoimmunity might be at the height of its activity. These spontaneously diabetic rats became normoglycemic following transplantation but, unlike the streptozotocin diabetics, all developed recurrent hyperglycemia within four days. Histologic examination of the transplanted islet cells at this time showed a heavy mononuclear infiltrate which closely resembled the original insulitis lesion, but was also similar to the findings in rejection of allogeneic islets.

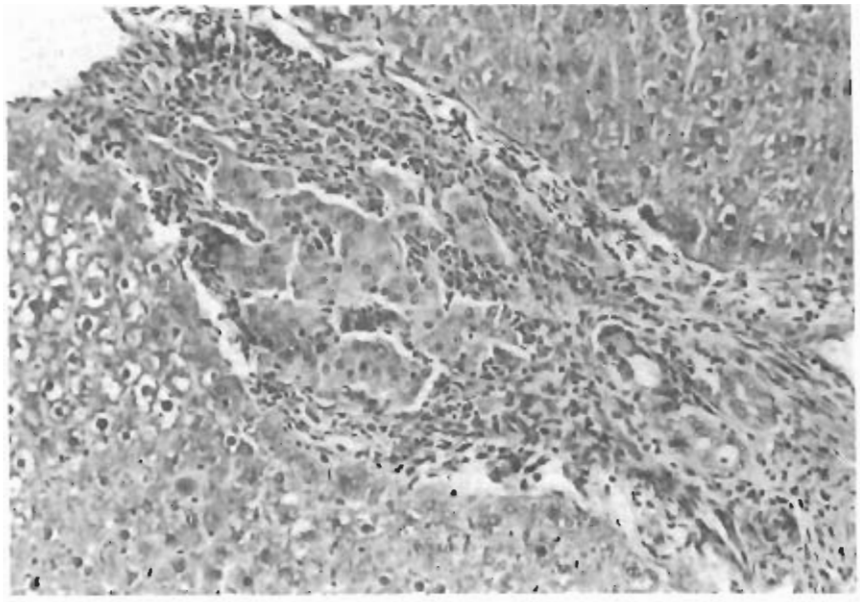

FIG. 3. In spontaneously diabetic "BB" rats tolerant of WF antigens, WF skin allografts survived permanently but WF islets transplanted to the liver invariably failed within 11 days. Liver biopsies such as this demonstrated lymphocytic infiltration of islets, which in this instance is attributed to autoimmune destruction rather than rejection (H & E ×200).

The more rapid recurrence of hyperglycemia following transplantation in naturally occurring diabetes as compared with the artificially induced condition, which in some islet cell transplants were actually permanently successful, strongly suggested that islet cell failure in the spontaneous diabetes occurred on the basis of recurrence of the original disease process rather than rejection.

We considered the possibility that an autoimmune process, which at its height would destroy either native islet cells or transplanted ones, might with time, diminish to low levels or even disappear completely. Therefore, the results of islet transplantation were ascertained in "BB" rats which, rather than being acutely diabetic, had the onset of their disease many weeks before. Two such rats were maintained on insulin therapy for five and three months after becoming hyperglycemic. Each was then transplanted with islets from normoglycemic "BB" rats of the nondiabetes-prone line. In neither of these animals did hyperglycemia rapidly recur. Instead, normoglycemia lasted for three months in the first instance (until death of the animal) and for four months in the other which eventually developed recurrent hyperglycemia. In three additional instances, diabetic rats which had remained hyperglycemic despite a 30 day course of ALS therapy, received an islet cell transplant 60 days after the original onset of the disease. They also had prolonged function of transplanted islet cells remaining normoglycemic for four-six months until their eventual deaths.

The role of rejection in failures of some allogeneic islet transplants could not be completely excluded. Therefore, WF islet transplants were studied in "BB" rats which had been rendered tolerant of the prospective islet donor strain (Fig. 2). Tolerance induction was accomplished by inoculation of the prospective recipients with 50×10^6 WF bone marrow cells during the first 24 hours of life. The subsequent permanent acceptance by these rats of WF skin allografts confirmed the tolerant state. Six tolerant "BB" animals which had not become diabetic in >150 days and, thus, were presumed to lack autoimmunity, were treated with streptozotocin to induce hyperglycemia and then transplanted with WF islets. As expected, the islets were not rejected and permanent normoglycemia ensued in all. Seven other tolerant "BB" rats which became *spontaneously* diabetic also received transplanted islets from WF donors. In contrast with the successful result in streptozotocin diabetics, these transplanted islet cells were capable of sustaining normoglycemia for only 1–11 days prior to recurrent diabetes, which, in these tolerant hosts, could not be attributed to rejection. In two other instances rats which had been rendered tolerant of either BN or ACI antigens also destroyed subsequent transplanted islet cells of these donor strains within two weeks time. In all cases of recurrent diabetes, a histologic picture of insulitis was found in the transplanted islets (Fig. 3).

Evidence of Immunodeficiency in "BB" Rats

"BB" rats which were derived from diabetic parents (even though they never became hyperglycemic) were often noticed to be underweight and generally less robust from birth than members of the nondiabetes-prone subline of "BB" rats in our colony. Members of the diabetes-prone line appeared to have an increased susceptibility to mycoplasma and other pathogens endogenous to our animal colony, and their mortality

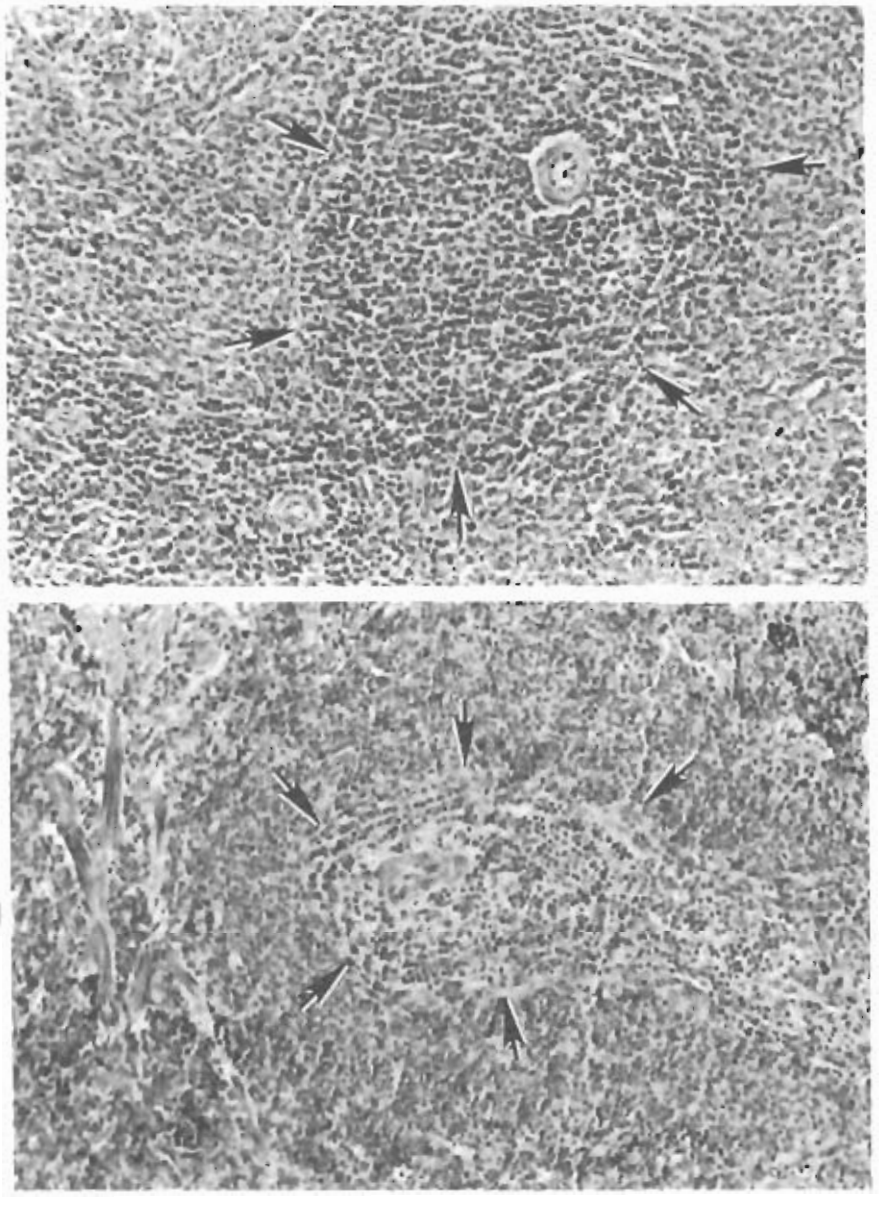

FIG. 4. The periarteriolar lymphatic sheath (PALS) in the spleen (delineated by arrows) is densely populated with small T lymphocytes in the normal rat (a, top). In a diabetic "BB" rat (b, bottom), the PALS region is characteristically acellular, providing evidence of T cell deficiency (H & E ×160).

rate from pneumonia and other infections was high, whether they were diabetic or not, as compared with that of other strains of inbred rats, or as compared with our nondiabetes-prone "BB" subline. There also appears to be a high incidence of malignant tumors in "BB" rats. Two malignant lymphomas (a tumor unusual in rats) have been found in members of our "BB" colony, a finding also reported by Kalant and Seemayer.[14] These findings led us to consider the possibility that diabetes-prone "BB" rats could be suffering from an immunologic deficiency disease which increased their susceptibility to infections, tumors and possibly to diabetes.

White blood cell counts were done on peripheral blood of "BB" rats and found to be in the normal range. However, thoracic duct lymph in ten "BB" rats from the diabetes-prone line was found to be low in total lymphocyte counts, as compared with lymph from either nondiabetes-prone "BB"s or from ACI, BN or WF rats, the range of cell counts in lymph of ten diabetic or diabetes-prone rats ranging from 5–60% of the count in five normal rats. Lymph nodes and spleen were also low in total lymphocyte counts. Histologic sections of these tissues revealed the additional important finding that the anatomic areas usually associated with high concentrations of T cells[15] were relatively acellular. Thus, in the spleen, the periarteriolar lymphatic sheath and in lymph nodes the paracortical regions were underpopulated by small lymphocytes (Fig. 4).

The above findings were highly suggestive of immunologic deficiency, and especially of a T cell deficiency, both in diabetic "BB" rats and also in young "BB"s with a genetic background, suggesting that they were likely to become diabetic. We examined one index of T cell function; skin graft rejection. Nine normoglycemic members of the diabetes-prone "BB" line all rejected MHC incompatible ACI skin allografts within 12 days. However, MHC compatible WF skin allografts were rejected in an indolent fashion with about 50% surviving more than 100 days.

We also examined the possibility that the immunologic disorder in "BB" rats could be improved or corrected with a resultant decrease in susceptibility to various diseases, including diabetes. Reconstitution of a more normal immune system in "BB" rats was attempted by treating them with bone marrow from normal rats of other strains. To avoid rejection of the transplanted allogeneic bone marrow it was administered to neonatal "BB" rats within 24 hours of their birth when immunologic immaturity could be relied upon to prevent rejection of the transplanted marrow cells. An additional expected outcome of this procedure was the development of immunologic tolerance of the marrow recipients to other tissues of the donor strain. Documentation of this tolerant state was obtained by permanent acceptance by the marrow recipients of skin allografts of the same donor origin, a procedure which also confirmed successful engraftment of the bone marrow inoculum in any individual rat.

A total of 14 litters of diabetes-prone "BB" rats were inoculated with 50×10^6 bone marrow cells from WF or (ACI × "BB") F_1 donors. In all cases, half of the members of the litters were inoculated, while the remaining rats were left untreated as controls. In almost all cases the inoculated members fared significantly better than their untreated littermates. They had a generally healthier appearance than their untreated littermates, although this was not reflected in their early growth pattern. In fact, the mean weight at eight weeks was slightly lower in inoculated rats (164.5 g) than in the untreated controls (177.9 g), perhaps reflecting nonspecific trauma of the inoculation (or very mild graft versus host reactions). Nevertheless, the vulnerability to pathogenic micro-organisms appeared to be strikingly greater in rats which had not been treated with neonatal bone marrow and many of them died of infections such as pneumonitis. In recipients of WF bone marrow, none of the 33 nondiabetic members of nine litters died in less than six months, the mortality rate being 9.1% at 12 months, and 21.0% at 18 months. In contrast, 16 nondiabetic *noninoculated* controls had a much higher mortality rate of 12.5% at six months, 43.6% at one year and 81.3% at 18 months, the differences at each interval being highly significant ($p < 0.01$). If deaths in diabetic rats had not been excluded from consideration in calculation of mortality rates, the difference would have been even greater, since diabetes was associated with a very high mortality rate and occurred much more frequently in rats which had not received bone marrow transplants. Recipients of MHC incompatible marrow (ACI × "BB") F_1 also appeared to have a better chance of surviving than untreated controls.

Examination of the lymphoid tissues of marrow recipients, as compared with those of the control groups, suggested that the lesser mortality rate in the former group might be on the basis of a more normal immune system. The lymph nodes and spleen of marrow recipients showed an increase in cellularity of T cell-dependent areas, which were relatively empty in the untreated control rats. There was also an increase toward normal of the total numbers of lymphocytes in lymph nodes and in thoracic duct lymph.

Evidence was also obtained that the neonatal marrow treatment improved function of the immune system. Eighteen untreated "BB" rats were grafted with skin from WF donors (compatible with "BB" recipients at the MHC but differing by minor transplantation antigens). These grafts were chronically rejected (36–210 days, median 74.0 days). On the other hand, six "BB" rats which had received ACI bone marrow at birth rejected WF skin allografts significantly more rapidly, in 17–20 days, median 17.1 days; ($p < 0.01$). A likely explanation was that the chimeric population of ACI cells at least partially compensated for the "BB" rats' own immunoincompetent state.

A somewhat unexpected finding of equal or greater importance was that the bone marrow recipients had a strikingly reduced incidence of diabetes, as compared with their untreated littermates (Table 1). This was particularly evident when WF marrow was given to half the members of each of nine litters in which a high incidence of diabetes was expected because one or both parents were diabetics. Of 39 untreated rats, 22 (56.4%) became diabetic. Seven deaths from infection in this group during the first few months of life may have prevented recognition of an even higher incidence of diabetes. However, in 37 animals which received neonatal bone marrow, only four (10.8%) became diabetic, a highly significant difference ($p < 0.001$). In three of the litters in which both parents were diabetic, all untreated rats became diabetic (a total of 14) while all marrow recipients (totalling 14) remained normoglycemic.

When (ACI × "BB") F_1 was the donor strain it also appeared that transplanted bone marrow discouraged

TABLE 1. *Incidence of Diabetes in Members of Individual Litters of "BB" Rats Inoculated with WF Bone Marrow and in Noninoculated Littermates*†

Parental Background*	Marrow Recipients	Untreated Littermates
D × D	0/4	4/4
D × D	0/6	6/6
D × D	0/4	4/4
D × D	0/3	0/1
D × D	1/2	0/1
D × D	0/3	2/5
D × D	3/6	2/7
D × ND	0/5	3/7
D × ND	0/4	1/4
Total	4/37 (10.8%)†	22/39 (56.4%)

* D = Diabetic, ND = Non Diabetic.

† The difference in incidence of diabetes was highly significant ($p < 0.01$).

‡ The protective influence of neonatal inoculation of 50×10^6 WF bone marrow cells on development of diabetes in highly susceptible "BB" rats, is shown.

development of diabetes, with three of 28 (10.7%) of marrow recipients becoming diabetic, as compared with five of 20 (25%) in the control group. However, this was not statistically significant ($p > 0.5$).

The explanation of the reduced incidence of diabetes in recipients of normal marrow remains to be elucidated. It seems likely that it depends on the persistance of cells of the allogeneic bone marrow or their descendents. In any case, the protection is not transmitted to subsequent generations. The incidence of diabetes in 239 offspring of pairs of untreated nondiabetic "BB" rats in our colony is 5.4%. However, in three instances, litters born of two nondiabetic marrow recipients have had a more than 50% incidence of diabetes, indicating that the heritable trait remains present in the marrow treated animals, despite their failure to become diabetic.

Discussion

Relatively slow progress has been made in defining the cause of diabetes mellitus. However, in the last few years there have been strong suggestions that the immune system and/or viral infections may play a central role.[16] The following evidence of this has been found in human insulin dependent diabetics: 1) susceptibility to the disease appears to be linked to certain MHC specificities such as B8/DW3 and B15/DW4;[17] 2) antibodies directed against islet cell antigens are present in many patients with diabetes, especially early in the course of the disease;[18] 3) mononuclear infiltration of the pancreatic islet cells is often present in patients dying soon after the onset of the disease;[19] and 4) Coxsackie virus B4 has been isolated from the pancreas of one young human diabetic.[20] Since experimental approaches to the etiology in man are difficult or impossible on ethical grounds, an animal model closely resembling the human disease would greatly facilitate such studies. The "BB" rat appears to be the best such model available. Our exploration of the etiologic possibilities is still in progress, but some interesting findings have already emerged.

In man, the MHC is known to be involved in susceptibility to diabetes and our genetic studies suggest that this may, likewise, apply to "BB" rats. Thus, not only are these rats uniformly homozygous for RT1[u] but all four members of two backcross populations which have so far developed the disease have also been found to be homozygous for this complex. The failure of the disease to occur in 200 rats of F_1 populations, along with the observation that it does not uniformly occur in inbred "BB" rats in which both parents are diabetic, and is only very rarely encountered in the offspring of F_1 rats backcrossed to "BBs", appears to indicate that its inheritance is polygenic with incomplete penetrance.

The fact that not all "BBs" which apparently have genetic factors predisposing them to the disease actually become diabetic could indicate that environmental factors are also important (although as in the case with many human identical twin pairs, where concordance is only 50%,[21] the environmental factors appear to be uniform in our colony). The environmental factor most frequently considered in man is viral infection. In this regard we have studied the pancreas and other tissues from more than 20 "BB" rats, either acutely diabetic, or presumed likely to be prediabetic, and have discovered evidence of viral infection in none. However, since the identity of the virus for which we are searching is unknown, these studies remain inconclusive.

The autoimmune nature of diabetes in "BB" rats, which was first suggested by the insulitis lesion itself, now appears firmly established. Our own successful use of immunosuppression and the similar experience of Like et al. in promoting healing of the insulitis lesion and reversal of hyperglycemia provides further evidence of an autoimmune origin.[13] Even more convincing in this regard is the recurrence of the disease in our ALS-cured rats after immunosuppression was stopped. Passive transfer of the disease with putatively immune serum or lymphoid cells would be the *sine quo non* for demonstration of its autoimmune nature. Our evidence in this regard is negative with regard to serum, in which we also failed to find evidence of islet cell antibodies. Preliminary evidence was obtained for passage of insulitis to "BB" rats of the nondiabetes-prone line by inoculation with lymphoid cells from acutely diabetic rats. However, larger numbers of experiments will be required to substantiate this finding.

The best evidence, thus far, for autoimmunity in

"BB" rats is provided by the islet transplantation studies. The rapid destruction of allogeneic "BB" islets from nondiabetic donors, when transplanted to acutely diabetic rats with the spontaneous disease, was statistically quite different ($p < 0.001$) than the much longer islet transplant survival in rats with hyperglycemia artificially induced with streptozotocin. Nevertheless, since rejection by the nonimmunosuppressed outbred rats is a theoretical possibility, it is reassuring that the same result was obtained in the tolerant animals where the design of the experiment excluded rejection as a possibility. Thus, this experiment provides conclusive evidence that the original disease process is responsible for the destruction of the transplanted islets.

The finding that islet cells transplanted to spontaneous "BB" diabetics of long standing are less likely to be destroyed by autoimmunity than ones transplanted in the acute phase of the disease, is encouraging for the possible success of islet transplantation in humans with long standing disease. That "BB" rats are immunodeficient might have been predicted from their increased susceptibility to infection and their high mortality rate which has been recognized by ourselves and others. The observation that lymphoid organs from the diabetic-prone "BB" line are relatively empty of T lymphocytes, which has not previously been reported, strongly suggests a specific defect in the immune system as does the impaired capacity of "BB" rats to reject MHC compatible WF skin allografts. Using serological markers we have also found that BB rats of the diabetes prone line have only about half as many T lymphocytes as normal rat strains. Restoration of normal cellularity to T cell compartments by neonatally administered bone marrow seems likely to be responsible for both the decrease in mortality rate from infection and restoration of capability to mount a prompt skin allograft response, which were observed.

An unexpected finding which may be of paramount significance is that the incidence of diabetes is strikingly less in "BB" rats which have received bone marrow from normal allogeneic donors. The mechanism of this phenomenon remains unknown. Several possibilities are: 1) deletion of a forbidden clone of host lymphoid cells responsible for causing autoimmune diabetes; 2) restoration of a clone of cells absent in "BB" rats and important in combating a yet to be identified diabetogenic virus; 3) correction of an imbalance in lymphocyte subpopulations such as deficiency in suppressor cells which might be crucial to aborting an autoimmune response to islets. It should be possible to devise experiments using the "BB" rat to explore each of these etiologic possibilities.

It seems likely that some of this information might have relevance to the origin of human insulin dependent diabetes. It is of note that much evidence already exists suggesting that human diabetics are deficient in their immune response.[22] Although it has usually been assumed that this is the result of their disease it is not at all inconceivable that this defect may, instead, be an important factor in causing diabetes. If so, some type of immunologic manipulation (such as we have shown to be effective in prevention of diabetes in "BB" rats) may eventually be worthy of trial in humans known to be genetically predisposed to the disease.

Acknowledgments

Thanks are due to Dr. P. Thiebert for providing a breeding nucleus of "BB" rats; George Sawchuck, Mina Naji, James Markmann, Gustava Black and Brigitte Koeberlein for excellent technical assistance, and to Frances Ramirez for the preparation of the manuscript.

References

1. Banting FG, Best CH. The internal secretion of the pancreas. J Lab Clin Med 1922; 251–266.
2. Report of the National Conference on Diabetes. Current Status and Future Directions. Appendix to the Third Annual Report of the National Diabetes Advisory Board. April 1980. United States Dept. of Health and Human Sciences, Public Health Service. NIH Publication no. 80-2073.
3. Renold AE. Spontaneous diabetes and/or obesity in laboratory rodents. *In* Levine R, Luff R (eds) Advances in Metabolic Disorders. New York, Academic Press. 1968; 3:49–84.
4. Geritsen GC, Dulin WF. Characterization of diabetes in the Chinese hamster. Diabetologia 1967; 3:78–84.
5. Nakhooda AF, Like AA, Chappell CI, et al. The spontaneously diabetic Wistar rat (the "BB" rat) Studies prior and during development of the overt syndrome. Diabetologia. 1978; 15:199–207.
6. Götze D. The major histocompatibility complex of the rat Rt-1. I. The serological characterization of the MNR haplotype ($RT1^m$), in regard to the cross-reacting haplotypes $RT1^a$, $RT1^c$, and $RT1^b$. Immunogenetics 1978; 7:491–506.
7. Bottazzo GF, Florin-Christensen A, Doniach D. Islet cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. Lancet 1974; 2:1279–1283.
8. Wilson DB, Marshak A, Pierson GR, Howard JC. Specific selection of cytotoxic effector cells: The generation of cytotoxic T cells in rat thoracic duct lymphocyte populations positively and negatively selected for reactivity. J Immunol 1976; 116:1684.
9. Billingham, RE. *In* Transplantation of tissues and cells. Wistar, Philadelphia, 1971. p. 87.
10. Reckard CR, Barker CF. Transplantation of isolated pancreatic islets across strong and weak histocompatibility barriers. Transplant Proc 1973; 5:761–763.
11. Naji A, Reckard CR, Ziegler MM, Barker CF. Vulnerability of pancreatic islets to immune cells and serum. Surg Forum 1975; 26:459–461.
12. Naji A, Silvers WK, Plotkin SA, et al. Successful islet transplantation in spontaneous diabetes. Surgery 1979; 86:218–226.
13. Like AA, Appell MC, Williams RM, et al. Spontaneous diabetes mellitus, reversal and prevention in the "BB"/w rat with antiserum to rat lymphocytes. Science 1979; 206:1421–1423.
14. Kalant N, Seemayer T. Malignant lymphomas in spontaneously diabetic rats. N Engl J Med 1979; 300;737.
15. Anderson AO, Reynolds Ja. Adjuvant effects of the lipid amine CP = 20. 961. J Reticuloendothel Soc 1979; 26:667–680.
16. Craighead JE. Current view on the etiology of insulin-dependent diabetes mellitus. N Engl J Med 1978; 299:1439–1445.
17. Cudworth AG, Woorow JC. Genetic susceptibility in diabetes

mellitus. Analysis of HLA association. Br Med J 1976; 2:846–848.
18. Nerup J, Lernmark A. Autoimmunity in insulin dependent diabetes mellitus. Am J Med 1981; 70:135–141.
19. Gepts W, LeCompte PM. The pancreatic islets in diabetes. Am J Med 1981; 70:105–115.
20. Yoon JW, Austin M, Onodera T, Notkins AL. Virus-induced diabetes mellitus, isolation of a virus from the pancreas of a child with diabetic ketoacidosis. N Engl J Med 1979; 300:1173.
21. Barnett AH, Eff C, Leslie RD, Pyke DA. Diabetes in identical twins. A study of 200 pairs. Diabetologia 1981; 20:87–93.
22. Handwerger BS, Fernandes G, Brown DM. Immune and autoimmune aspects of diabetes mellitus. Human Path. 1980; 11:338–352.

Discussion

Dr. Olga Jonasson (Chicago, Illinois): To corroborate a bit of Dr. Barker's finding, it has been known for a number of years that there is an association of juvenile-onset diabetes in humans with human HL-A. It is not a universal finding, but it certainly seems that certain HL-A phenotypes do predispose individuals to juvenile-onset diabetes.

For instance, the combination of HL-A B8 and B15 increases the relative risk in individuals who have both of these HL-A antigens from 18 to 20 times. In accord with what we are learning about the major histocompatibility complex (MHC) in humans, that genes determining immune responsiveness and predisposing toward immune phenomena are located in the MHC, the finding that in these "BB" rats a similar genetic predisposition exists implicates the genes in the areas of immune responsiveness, so well described by Benacerraf and others in their Nobel Prize-winning work this past year.

Dr. Anthony P. Monaco (Boston, Massachusetts): I would also like to point out that the concept of viral infection is relevant to human juvenile diabetes, along with the HL-A markers, as identified by Dr. Jonasson. Large clinics which have a large experience with new juvenile-onset diabetes identify the increased numbers of these kinds of patients with seasonal variations, especially in the Northeast during the "flu season." They consider this as possible evidence of a virus-induced disease that could lead to destruction of the islets by some type of autoimmune mechanism. There are people who, with the kind of work presented by Dr. Barker, believe it is now appropriate to consider a transient treatment of acute juvenile diabetes with some form of immunosuppression, in the hope of modifying the insulitis that no doubt takes place in these patients.

Dr. James V. Maloney (Los Angeles, California): Dr. Barker, let me, as a person who knows nothing about this field, ask you a question. We are all excited about Boyers' work in genetic engineering and about Millstein's work on hybridomas.

It is conceivable that in the foreseeable future, this problem of immunosuppression will be addressed by the insertion of genetic information into human T cells?

Dr. Clyde F. Barker (Closing Discussion): I shall try to answer Dr. Maloney's question first. It is a difficult one, and I am certainly no expert in that field.

It seems to me that the most difficult problem with accomplishing what you have suggested, that is, "programming" nonislet cells of a diabetic's own body to make insulin, thus avoiding the need for transplantation and the risk of infection, is that, although certain cells and bacteria have been induced to produce insulin *in vitro*, one of the most important parts of the control system for diabetes is the ability of the islet cells to sense the glucose level and secrete the appropriate amount of insulin almost instantaneously. An intact pancreas seems to be able to begin to secrete insulin even before the onset of an elevation in the blood glucose level.

It is hard to conceive that cells other than pancreatic islet cells will be able to do this. However, this general approach is one that may hold great promise for palliation of diabetes.

To comment on the other two discussants' remarks, I did not have time in the oral presentation to discuss the genetics of this animal model of diabetes, but it seems similar in this regard to what we know about the genetics of human diabetes. Both diseases appear to be linked to the major histocompatibility locus. In the BB rat, our back-cross population, in which F1 hybrids between BB rats and nondiabetic strains are then back-crossed to BB rats, we have had four animals, of about 30, which became diabetic. All of these animals were homozygous for the $RT1^u$ allele. So the diabetes seems to be MHC linked.

With regard to the infection issue raised by Dr. Monaco, one of our disappointments is that we have not been able to find a virus in the BB rat, since we continue to suspect that there may be one there, which is important in the pathogenesis of this autoimmune process. However, we have not been able to isolate a virus, perhaps because we are looking for an unknown virus whose culture requirements are not known. It is unlikely that the rat virus, if there is one, is the same etiologic agent that might cause the disease in humans.

The Coxsackie B-4 virus, which is one of the leading candidates for causing the disease in humans, has been inoculated into BB rats. It fails to replicate in this species. Therefore, I believe that finding the diabetogenic virus, if one exists, may be a complicated task. I would not be surprised if it is there.

With regard to the current therapeutic trials of immunosuppression of newly diabetic patients as suggested by Dr. Monaco, I suspect that this may ultimately prove to be effective therapy, just as it is in rats. Nevertheless, we should approach it with caution, since it might also be extremely dangerous. We know from immunosuppression in other settings that in the setting of an acute viral infection, immunosuppression can be dangerous. This deserves careful consideration before clinical trials of immunosuppression are begun.