The Cellular Stimuli for the Rejection of Established Islet Allografts

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SUMMARY

Previous work from our laboratory has indicated that the transplantation of pancreatic islets is a feasible approach to the problem of diabetes. A major obstacle to transplantation is presented by passenger leucocytes, which contaminate the preparations and can lead to the prompt rejection of fresh islets. We have extended our previous studies on the rejection of islet allografts by challenging transplanted animals with enriched lymphoid cell populations prepared from animals both syngeneic to the transplanted islets and third party. Rapid and complete rejection was observed when the challenge peritoneal exudate cell population was syngeneic with the transplanted islets; rejection was determined by both functional and histologic criteria. Peritoneal exudate cells from a third-party rat strain induced delayed and variable effects upon the function of the transplant. In contrast, splenic T-cells were capable of inducing rejection, regardless of the strain of origin, though the time course of T-cell-induced rejection was slower than that observed by syngeneic peritoneal exudate cells. Finally, splenic B-cells completely failed to induce rejection.

Our data indicate that at least two mechanisms exist by which the rejection of islet allografts may be triggered. The first is a haplotype-specific mechanism initiated by a cell type present at high frequency in peritoneal exudate cells; these are probably macrophages. The second mechanism is initiated by immunocompetent T-cells; this mechanism shows no haplotype specificity. We suggest that both macrophages and T-cells must be considered when devising protocols for the removal of passenger leucocytes from allografts. DIABETES *30*:242–246, March 1981.

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The transplantation of pancreatic islets is a promising solution to the problem of diabetes mellitus. We have previously reported[1] successful pancreatic islet transplantation between fully allogeneic strains of rats, in which donors and recipients differed at multiple genetic loci, including the major histocompatibility complex[1] and, more recently, xenogeneic transplantation.[2] Successful reversal of the streptozotocin-induced diabetes required both the administration of antilymphocyte serum to the recipient and the prior culture of the donor islets.[1,2] On the basis of our demonstrated need for the culture period, we proposed that during this time in vitro, islet cells had a demonstrably retained function, but that there might have been the loss of contaminating "passenger" leucocytes with the capacity to stimulate rejection. Our observations and interpretations were entirely consistent with those of Lafferty and Talmage[3–5] in their murine thyroid transplantation model. We further showed[6] that rejection of established islet allografts could be induced by the injection of peritoneal exudate cells (PEC), syngeneic to the islet donor, and suggested that this might be analogous to the passenger population, present in fresh islets, which would lead to the rapid rejection of uncultured islets.

The experiments that we report here were designed to extend our investigations of the cell type or types capable of inducing rejection. If rejection of established islet allografts by injection of peritoneal exudate cells (PEC) was, indeed, mimicking the role of passenger leucocytes in fresh grafts, could we gain an insight into the nature of the passenger cell(s) by challenging grafted animals with enriched or purified cell populations and observing the effects of these upon islet function? Our results indicate that there are, potentially, two types of passenger leucocytes: T-cells, which initiate a nonspecific rejection, and macrophages, which elicit a specific rejection phenomenon. Interestingly, B-cells show no evidence of fulfilling any active role in inducing rejection, even though these cells have been shown to bear Ia antigens.[7]

MATERIALS AND METHODS

Animals. Male Lewis rats (AgB[1]) (Simonson Laboratories and Charles River Biological Company) were used as recipients, and male Wistar-Furth rats (AgB[2]) (Microbiological Associates, Walkersville, Maryland) were used as islet donors. Spleen cells or peritoneal exudate cells (PEC) for challenge were taken from Wistar-Furth or Buffalo rats (AgB[6]) (Microbiological Associates). The Lewis rats were made diabetic by the i.v. injection of 65 mg/kg streptozotocin,[8] and the diabetic status was monitored daily by measuring urine volume, urine glucose, and body weight. Plasma glucose concentration was measured twice a week subsequent to challenge with enriched cell populations.

Isolation and culture of islets. Islets were isolated by the collagenase technique[9] and were separated by centrifugation on a Ficoll gradient.[10] Clean islets were picked manually using a dissecting microscope under green light.[11] Isolated islets were cultured for 7 days at 24°C in a humidified atmosphere of 5% CO_2 in air. The medium employed for culture was CMRL-1066 (Microbiological Associates), supplemented with 100 μg/ml streptomycin and 10% fetal bovine serum (Microbiological Associates). The glucose concentration of the medium was adjusted to 1.5 mg/ml.

Transplantation of islets. Islets were collected and 1200–1500 islets were transplanted into the liver of a diabetic recipient through the portal vein.[1] At the time of transplantation, 1 ml of antilymphocyte serum (rabbit anti-Wistar-Furth lymph node cells, Microbiological Associates) was given to the recipient intraperitoneally.

Preparation of cell suspensions for challenge. Spleen cells were obtained by teasing the organ apart gently with forceps. Erythrocytes were lysed with Tris-buffered ammonium chloride[12] on ice. The medium used for preparation and purification of cells was RPMI-1640 (Microbiological Associates), supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and HEPES-buffer (20 mM). T-cell-enriched populations were obtained by passage of spleen cells over two sequential nylon wool columns.[13] Splenic B-cells were selected by using anti-immunoglobulin-coated plates, as described by Wysocki and Sato.[14] The anti-immunoglobulin was prepared from anti-rat immunoglobulin serum (Gateway Immunosera, Cahokia, Illinois) by ammonium sulfate precipitation, and adjusted to 25 μg/ml in Tris-HCl buffer (50 mM, pH 9.5) for coating the plates. Spleen cells suspended in phosphate-buffered saline containing 5% fetal calf serum were applied to the dishes and incubated for 75 min at 4°C. Nonadherent cells were removed, and adherent cells were recovered by flushing. PEC were harvested 3 days after i.p. injection of 15 ml of 3% or 10% thioglycollate broth (Difco, Detroit, Michigan).

Characterization of cell populations. B-cells were identified by fluorescence microscopy after staining with fluorescein isothiocyanate-conjugated goat anti-rat immunoglobulin under noncapping conditions.[15] Phagocytic cells were identified by ingestion of latex particles (Dow Chemical Co., Indianapolis, Indiana). Only cells containing four or more particles were counted as positive.

Induction and criteria of rejection by cellular challenge. Lewis recipients were challenged by the i.v. injection of viable splenic T-cells, splenic B-cells, or PEC. The viability of the cells was determined by trypan blue exclusion, and the number of cells injected was expressed as viable cells. The methods used for separation of T- and B-lymphocytes did not affect the viability of the cells. The time from the transplantation to challenge with lymphoid cells ranged from 40 to 180 days.

Transplanted and challenged Lewis rats were kept in metabolic cages. Rejection was considered to have been initiated when the urine glucose exceeded 2 g/day or a urine volume of 20 ml/day. Complete rejection was defined as the time at which urine glucose content on 3 out of 5 consecutive days reached the pretransplant level. Determining the time for initiation and completion of rejection made it possible to evaluate the pattern and rate of rejection for each recipient.

Histologic findings. At autopsy, the liver was sliced into 2-mm-thick pieces, and alternating pieces were fixed in Bouin's solution. The tissue was embedded in paraffin, and at least one section was cut from each piece. The sections were stained with hematoxylin and eosin, and aldehyde fuchsin.[16]

RESULTS

In our previous studies,[6] we found that in the Wistar-Furth to Lewis system, rejection could be induced by multiple challenges with Wistar-Furth PEC. Preliminary experiments (data not shown) established that the threshold number of PEC required to induce rejection by a single cell challenge was approximately 1.5×10^7. For unfractionated WF spleen, 6×10^7 cells induced clear and rapid rejection, and we elected to use this cell number, 6×10^7, for challenges with enriched lymphoid populations.

Characterization of enriched cell populations. The markers employed were surface immunoglobulins (sIg) for B-cells and both morphology and the ingestion of latex particles for macrophages (see MATERIALS AND METHODS). The data in Table 1 show the mean values of these two markers, determined in a large series of separations. Unfractionated spleen cells contained 49% sIg^+ cells; this frequency could be reduced to 3% by passage over two sequential nylon wool columns and increased to 77% by positive selection on anti-Ig-coated plates. The frequency of phagocytic cells was decreased by both these procedures: nylon wool passage almost completely eliminated latex positive cells (in some experiments their frequency was reduced to <0.1%), and their frequency in the B-cell populations was reduced fourfold. It should be noted that no positive marker for T-lymphocytes was available to us at the time, and that their frequency in unfractionated spleen cells

TABLE 1
Characterization of lymphoid cell populations from WF rats

Cells	sIg positive (%) (B-cells)	Latex positive (%) (macrophages)
Spleen cells	49	4
Splenic T-cells*	3	<1
Splenic B-cells†	77	1
Low latex positive PEC‡	ND	<38
High latex positive	ND	>52

* Double-passed over nylon wool columns.
† Isolated with anti-Ig immunoabsorbent.
‡ Thioglycollate-induced peritoneal exudate cells (PEC).

TABLE 2
Rejection of established allografts (WF to Lewis) induced by WF lymphoid cells

Cell type	No. of animals challenged	Induction of rejection	Day of rejection: Initiation of rejection	Day of rejection: Time to reach pretransplant level
T-cells	4	4	7, 9, 10, 11	24, 51,* 56, 112
B-cells	4	0		No rejection
Low latex positive PEC	3	0		No rejection
High latex positive PEC	5	5	6, 7, 8, 9, 11	11, 11,* 15, 19, 36

* Animal died.

and the purity of the nylon wool-passed populations was assessed by determination of contaminating sIg^+ and latex-ingesting cells. While this may introduce an error, the frequency of T-cells was at least 96% (Table 1) thus, the results of challenging with T-cells are not called into question by this. Thioglycollate-induced PEC were employed as a source of macrophage-enriched populations for challenge. We found that the frequency of latex-ingesting cells in the PEC population harvested at 3 days could be significantly influenced by the concentration of the thioglycollate used for induction. The low latex-positive population (frequency of phagocytes $\leq 38\%$) was induced by injection of 3% (w/v) thioglycollate; the high latex-positive (frequency $\geq 52\%$) resulted from injection of 10% (w/v) thioglycollate. This disparity is very important, in that the two populations of PEC differed in their ability to induce rejection of established allografts (Table 2). Morphologic examination of the high latex-positive populations showed predominantly macrophages (range 72–89%), with variable contamination by polymorphonuclear leucocytes (range 4–26%) and lymphocytes (range 2–7%).

Differential ability of enriched lymphoid cell populations to induce rejection of islet allografts. A marked difference in the ability to induce rejection was observed when we compared the two populations prepared from spleen. T-cells gave rise to a rapid initiation of rejection, with an eventual return of urine glucose to pretransplant levels (Figure 1 and Table 2). B-cells failed completely to induce rejection: animals were monitored for up to 6 mo, during which time no signs of rejection were observed.

The marked difference in biologic effect between low and high latex-positive populations of PEC is also shown in Table 2. The low latex-positive population failed to induce rejection, despite the significant content of phagocytic cells. In contrast, the high latex-positive population induced rapid initiation of rejection, associated with the return of urine glucose to pretransplant levels more rapidly than that seen with T-cell challenge.

The marked differences in composition of splenic T and high latex-positive PEC populations, coupled with the difference in the length of time after challenge for the urine glucose to return to pretransplant levels, prompted us to examine whether there might be two distinct mechanisms by which rejection was being induced. To study this question, we employed lymphoid cell populations prepared from a third-party strain, the Buffalo rat.

Splenic T-cells and high latex-positive PEC populations were prepared from Buffalo rats, as described. Their characteristics were essentially the same as the Wistar-Furth populations, with respect to sIg^+ and latex-positive contents. However, upon challenge, very marked differences were observed between the two Buffalo populations. The T-cells induced rejection with a pattern as rapid as that shown with Wistar-Furth T-cells (Figure 1). In contrast, of the three animals challenged with Buffalo PEC, rejection was initiated in one animal at 8 days (employing the criterion of 2 g/day urine glucose), but the urine glucose did not reach the pretransplant level until 40 days. A second animal showed markedly delayed initiation, never returning to pretransplant levels, and the third animal remained aglycosuric, showing no signs of rejection at all. Comparison of the effects of T- and peritoneal exudate cell challenges, employing cells from Wistar-Furth and Buffalo rats, thus further strengthened our view that two mechanisms of rejection existed.

Histologic findings. Autopsies were performed on four recipients challenged with Wistar-Furth T-cells at 51, 257, 282, and 316 days after challenge. Although the urine glu-

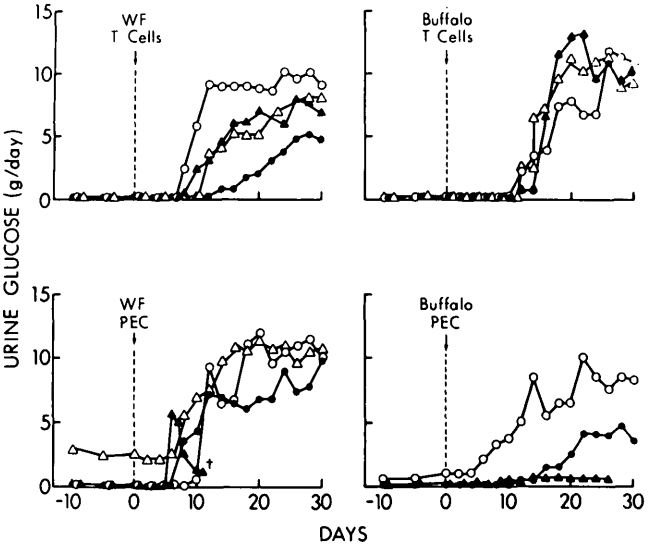

FIGURE 1. The effects of challenging Lewis rats bearing established islet allografts of Wistar-Furth origin with enriched cell populations of Wistar-Furth or Buffalo origin. Lewis recipients of Wistar-Furth islet allografts were challenged with 6×10^7 splenic T-cells or high latex-positive, thioglycollate-induced peritoneal exudate cells from donors syngeneic to the transplant (WF) or a third party strain (Buffalo). Urine glucose (abscissa) was monitored throughout; the data shown represent values obtained during the 10 days preceding and the 30 days succeeding challenge. Each of the symbols represent individual animals.

cose of these recipients had reached pretransplant levels long before the animal was killed, islets were found in the livers of all four recipients. The transplanted islets contained aldehyde fuchsin-positive cells as well as some negative cells. In some cases, aldehyde fuchsin-positive cells and liver cells mingled together, which suggested migration of cells in the liver. In some instances, lymphoid cells were seen to have infiltrated into the islets. Marked hypertrophy, with increased glycogen storage of the liver cells around the islets, reflected the exposure of these cells to high concentrations of insulin, which was released directly to the liver from the transplanted islets, and showed that functioning islets were present in that area.

Four recipients, challenged with B-cells, were autopsied. Three were killed 85, 290, and 367 days after challenge, and one died 318 days after challenge with B-lymphocytes. Three of these had well-preserved islets in the liver, and less cellular infiltration was observed than in the T-cell-challenged animals. Transplanted islets were not found in one recipient. This recipient had developed an insulinoma in the pancreas, presumably induced by streptozotocin injection,[17] and showed blood glucose concentration of 47 mg/dl at the time the animal was killed. The large amount of insulin secreted by the insulinoma cells may have caused atrophy of the transplanted islets.

Four recipients challenged with Wistar-Furth PEC were autopsied 11, 148, 233, and 234 days after challenge. No islets or liver cell hypertrophy were observed in the latter three recipients. Focal fibrotic areas, similar to the size of islets, were scattered throughout the liver. One recipient was killed for autopsy 11 days after challenge because of its poor general condition. Blood glucose concentration at the time of autopsy was 783 mg/dl. Islets still remained in the liver at this stage but show marked cellular infiltration. These histologic findings, then, support our hypothesis, based upon functional evidence, that at least two mechanisms exist by which the rejection of islet allografts may be triggered.

DISCUSSION

In this report, we have presented evidence, both functional and histologic, that the rejection of established pancreatic islet allografts may be mediated by two separate and distinct mechanisms. The first mechanism, which represents true and complete rejection, is that induced by PEC syngeneic to the islet donor. This is characterized by early onset, a rapid return of urine glucose to pretransplant levels, and a loss of islets from the recipient liver. A caution should be introduced here, since our sampling of the recipients' livers (alternate 2-mm pieces, limited sections from each piece for examination) does not permit us to claim complete elimination of all islets. However, we feel justified in claiming a marked reduction in their frequency, associated with the return to the diabetic state, following PEC challenge. In addition, challenge with PEC from a third party strain, Buffalo, induced variable and delayed effects that were quite clearly different from those induced by Wistar-Furth PEC. The variable response induced by Buffalo PEC may have been due to a low level contamination with T-lymphocytes, or possibly the Buffalo and WF strains may share some minor antigenic determinants that could induce a delayed rejection. The nature of the cell type in the Wistar-Furth PEC responsible for causing rejection of established Wistar-Furth allografts is indicated by comparing the effect of low and high content of latex-positive cells in the exudate: the former failed to induce rejection, while the latter were very potent. Since the ingestion of latex particles is a marker of macrophages, and the low and high latex-positive populations showed a sharp distinction in the induction of rejection, the correlation leads us to conclude that this mechanism of rejection is induced by macrophages present in the thioglycollate-induced peritoneal exudate population. These cells, in this model, would present antigens unique to the donor Wistar-Furth rats and would be capable of initiating the sequence of events that lead to islet rejection. Since fresh murine islets have been shown to bear antigenic specificities encoded in the K and D regions of the major histocompatibility complex[18] but not I-region products, the simplest interpretation is that Ia antigen-positive macrophages in the PEC population induce a population of T-cells (in the Lewis recipient) analogous to the Ia-reactive Lyl$^+$ helper cells for cytotoxic[19] and delayed-type hypersensitivity[20] effectors demonstrated in a variety of murine systems. As a consequence of the development of helper function, the effector cells for rejection would also be induced, leading to rejection of the established islets.

The second mechanism for the return to diabetic status is induced by T-cells. In contrast to the effects of challenge with PEC, no differences were observed between T-cells from Wistar-Furth and Buffalo rats. That is, no haplotype or strain specificity can be inferred from the data. In addition, although there was a clear return to diabetic status induced by Wistar-Furth T-cells, functioning islets persisted in the recipients' livers. A number of possible cellular mechanisms exist that might satisfactorily explain these observations. The first would be one in which the allogeneic, immunocompetent T-cells induce a graft versus host response in the recipient which would, in its turn, initiate a population of host effector T-cells. These effectors would be clearly different from those induced by PEC challenge, but capable of giving rise to the peripheral manifestations of diabetes without destroying the islets. Such effects have been described both through cell-mediated and antibody-mediated mechanisms. Van Schilfgaarde et al.[21] have recently shown amplification of the host response to cardiac and renal allografts, by a graft versus host-triggered mechanism. The second alternative would be an antibody-mediated mechanism. The introduction of Wistar-Furth or Buffalo T-cells into the Lewis recipient is precisely the protocol required to induce an allogeneic effect.[22] This has been shown to stimulate vigorous antibody production in both guinea pig[22] and murine systems.[23] Since the transplanted islets must, of necessity, still bear some Wistar-Furth alloantigens (otherwise they would presumably be completely resistant to rejection), they would provide the stimulating antigens for the clones of specific Lewis B-cells, and the allogeneic T-cells would provide an alternative pathway of T helper function. The antigens recognized by the antibody population are not easy to predict, though obvious candidates would be major histocompatibility products and, perhaps, insulin itself. Thus, one could postulate an interesting dual function of the antibodies: the enhancement of islet allograft survival coupled with either a decrease in the rate of insulin secretion or its

sequestration before reaching the periphery. The latter two alternatives are not mutually exclusive.

The complete failure of Wistar-Furth B-cells to affect islet allograft survival is also consistent with the two models that we have proposed. Although rat B-cells have been shown to bear Ia antigens,[5] it has also been shown that B-cells are incapable of stimulating mixed leucocyte responses in mice.[24] The mixed lymphocyte response is the in vitro analogue of Lyl$^+$ helper[25] T-cell function required for the generation of cytotoxic or delayed-type hypersensitivity effectors. Therefore, either B-cells and macrophages must differ in the Ia antigens that they express, or the B-cell membrane does not present Ia in the appropriate conformation to trigger a response or, finally, B-cells are able to present antigen but fail to produce a co-stimulatory factor[24] required for the response. In addition, B-cells do not function to induce an allogeneic effect, which is solely a property of T-cells.[23]

Our experiments, then, enable us to eliminate B-cells as being functionally important in the passenger leucocyte population responsible for the rejection of fresh islets. This is not to suggest that B-cells are completely neutral: Lauchart et al.[26] have shown that B-cells can induce active enhancement, and we feel that they may have a role in generating active suppression (I. M. Zitron et al., manuscript in preparation). It is equally clear that both T-cells and a population within the PEC (presumably macrophages) can induce the return to the diabetic state, and that the elimination of both of these must be the principal goals in procedures designed to remove passenger leucocytes from allografts.

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