Responses of Canine Lymphocytes to Allogeneic and Autologous Islets of Langerhans in Mixed Cell Cultures

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ABSTRACT Because successful allotransplantation of islets of Langerhans isolated by collagenase digestion has been difficult in many animal species, we asked whether isolated islet preparations might have tissue specific determinants conferring amplified immunogenicity in vitro. Lymphocyte proliferative responses ([^{3}H]thymidine uptake) were studied in beagle dogs in mixed culture combinations of lymphocyte vs. lymphocyte (MLC) and lymphocyte vs. islet (MLIC). In five MLC responder and five nonresponder pairs, peripheral blood lymphocytes of dogs A and B were used as responding cells, and dog B x-irradiated lymphocytes (Bx), x-irradiated (or nonirradiated) islets (BI), or hepatic cells (BH) were used as stimulating cells in primary and secondary reactions. For the secondary reactions, A + Bx, A + BI, or B + BI were incubated for 9 d (A'B, A'BI, B'BI, respectively) before addition of new stimulating cells.

The results showed that islets were autostimulatory, eliciting a tissue-specific lymphoproliferative response in a primary MLIC. Thus, B + BI reactivity was evident at 3, 5, and 7 d in primary culture, whereas collagenase-digested liver cells, or lymphocytes obtained from collagenase-digested lymph nodes did not stimulate autologous lymphocytes. A separate reactivity was observed in the allogeneic A + BI combination in MLC responder pairs, and the peak response of A + BI at 9 d was markedly greater than that of B + BI, suggesting the presence of major histocompatibility complex lymphocyte-defined locus determinants in the islet preparations, in addition to islet-specific determinants. A secondary reaction was observed if lymphocytes were primed with islets and challenged with islets (A'BI + BI or B'BI + BI), but not if they were challenged with lymphocytes (A'BI + Bx, B'BI + Bx) or hepatic cells (A'BI + BH, B'BI + AH). Furthermore, priming of lymphocytes with autologous islets (B'BI) led to exclusion of any reactivity against allogeneic lymphocytes, i.e., B'BI suppressed A + Bx, and B'BI also markedly suppressed phytohemagglutinin-stimulated lymphoproliferative responses. Experiments were performed that excluded the possibility that the insulin levels present in the MLIC, the presence of passenger lymphocytes in the islets, or the maintenance of islets in tissue culture for 1–7 d affected the observations. These results provide evidence for the existence of alloantigens as well as tissue-specific antigens on collagenase-isolated islets of Langerhans.

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INTRODUCTION

Transplantation of the endocrine pancreas has been the subject of increasing experimental and clinical study over the past several years. Although the vascularized organ allograft evoked most of the early interest (1), subsequent development of enzymatic digestion procedures to isolate islets of Langerhans from the exocrine pancreas has permitted transplantation of isolated islet tissue (2). However, recent studies suggest that survival of a vascularized pancreatic allograft is superior to that of an equivalent mass of allogeneic isolated islets, either partially or fully dissociated from the pancreas (3, 4). In general, dissociated tissue allografts are less readily accepted than immediately vascularized organs. This has been attributed to susceptibility to humoral immunity, due to the presence of many marginally viable cells in tissue implants dependent upon diffusion in place of an organized vascular supply (5), as well as the possibility of more efficient processing of antigen elaborated by dissociated tissue which lacks a "privileged site" that might otherwise be present in the vascularized organ.

In this report, we have examined islets of Langerhans, isolated from the beagle dog pancreas by collagenase digestion for their capacity to induce immune

recognition in vitro, i.e., lymphoproliferative responses in mixed lymphocyte-islet cultures (MLIC).[1] The experimental results provide evidence for the expression of alloantigens as well as tissue-specific antigens on isolated islets of Langerhans.

METHODS

Animals. Adult beagles purchased from Marshall Livestock Inc., North Rose, N. Y., and mongrel dogs conditioned for 4 wk in our laboratory kennels were used as donors of peripheral blood lymphocytes and pancreatic islets. In conducting the research described in this report, the investigators adhered to the Guide for Laboratory Animal Facilities and Care, of the Institute of Laboratory Animal Resources, National Academy of Sciences–National Research Council.

Isolated islet and islet cell preparations. The left, right, or both lobes of the canine pancreas were asceptically removed at laparotomy, leaving the head of the pancreas attached to the duodenum. For the secondary mixed lymphoislet cultures, two laparotomies were performed 10 d apart in the same donor and single pancreatic lobes were removed on each occasion. Islets were isolated by the collagenase enzyme digestion procedure originally reported for rat pancreas (6), modified for the more fibrous dog pancreas. In brief, after treatment with collagenase, the pancreatic digest was washed, and incubated for 18 h at 37°C in enzyme-free medium to allow islets to separate more completely from the remaining pancreatic tissue. The islets were then isolated by centrifugation on Ficoll density gradients and incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air, in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. Viable islets were hand-picked, using a dissecting microscope, and added to lymphocytes in MLIC. In some experiments, the islets were cultured for up to 7 d at 37° or 24°C before being added to lymphocytes in MLIC. Islets prepared in this manner appeared viable using phase-contrast microscopy and continued to secrete insulin in response to glucose challenge in vitro.

Further dissociation of islets was performed using 3 mM EGTA, followed by 0.1% trypsin (7). This treatment yielded a predominantly single cell preparation and allowed us to count the number of cells per islet: 2,050±310 (mean±SEM, $n = 6$ different groups of 200 islets). This value is similar to that obtained by morphometric methods (8).

In addition, single cell preparations were made from wedge sections of liver removed at laparotomy, and from popliteal and mesenteric lymph nodes, employing the collagenase enzyme digestion procedure used to isolate islets. The resulting cells were washed and cultured for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air, in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, before being used as stimulating cells in mixed lymphocyte cultures (MLC).

MLC and MLIC. The MLC in dogs has been previously described (9, 10). Briefly, triplicate microcultures were prepared in microtiter plates containing 10^5 responding and 10^5 x-irradiated (1,500 rad) stimulating peripheral blood lymphocytes isolated by density gradient centrifugation. Previous studies in our laboratory have shown that the canine MLC reactivity was optimized when these cell numbers were employed (9, 10).

For the MLIC, initial studies using 25, 50, 100, and 200 islets per 10^5 responding lymphocytes showed that the ratio of 100 islets ($\sim 2 \times 10^5$ cells) per 10^5 lymphocytes evoked the maximum lymphoproliferative response. 100 viable whole islets were hand-picked under the dissecting microscope and placed in triplicate in flat bottom wells in microtiter plates in 0.1 ml medium. To the islets were added 10^5 peripheral blood lymphocytes in 0.1 ml of RPMI 1640 medium supplemented with 15% heat-inactivated dog serum, penicillin-streptomycin, and glutamine. In some MLIC experiments, single islet cells were dissociated from whole islets and used as stimulating cells (2×10^5 cells) with 10^5 responding lymphocytes. In experiments with collagenase-isolated hepatic cells, or lymph node lymphocytes, single cell preparations of 2×10^5 cells were used as stimulating cells.

Both MLC and MLIC were incubated in a humid atmosphere of 7% CO_2 for 3, 5, 7, and 9 d in MLC, and 2, 3, 5, 7, and 9 d in MLIC, and pulse-labeled with 1 μCi of [^{3}H]thymidine for 18 h before processing with the Mash II microharvester (11) for liquid scintillation counting (Packard Instrument Co., Inc. Downers Grove, Ill.). The secondary MLC and MLIC were performed in triplicate by pooling several primary microcultures (10) after 10 d, and adding fresh complete media and fresh stimulating lymphocytes or islets into new microplates. Cultures were performed in triplicate and expressed as means for values with a variance <15%. The statistical significance of differences was determined by the Student's t test.

Glucose and insulin measurements. Separate MLC and MLIC cultures were set aside for determination of glucose and insulin concentrations in the culture media. After 4 h, and 3, 5, 7, and 9 d of culture, media and cells were removed from the microwells, the cells were pelleted by centrifugation at 300 g for 10 min, and glucose (glucose analyzer, Beckman Instruments, Inc., Fullerton, Calif.) and radioimmunoreactive insulin (12) were measured in the supernatant media.

RESULTS

Primary MLIC of MLC reactive pairs. Five pairs of animals were chosen in which the primary MLC reactivity of lymphocytes from the responder (A) vs. x-irradiated lymphocytes from the stimulator (Bx), as determined by previous assays, was strong (A + Bx > 9,000 cpm of [^{3}H]thymidine incorporation) compared with autologous control combinations (B + Bx or A + Ax < 500 cpm). Fig. 1A shows that islets stimulated both allogeneic and autologous lymphocytes to respond (A + BI and B + BI, respectively). The overall kinetics of these two MLIC reactions were different from each other and from the allogeneic MLC reaction. Thus, the proliferative responses of lymphocytes to islets (A + BI, B + BI) appeared earlier than the one to lymphocytes (A + Bx), and the peak response in the autologous MLIC combination (B + BI) occurred at 5–7 d, whereas the allogeneic MLIC combination (A + BI) exhibited a peak response at 9 d, and this was lower than that observed in the allogeneic MLC combination (A + Bx) ($P < 0.005$ in five pairs studied). Further dissociation of the whole islets to the single cell suspensions did not alter the autologous or allogeneic MLIC reactions. On

[1] *Abbreviations used in this paper*: H, hepatic cells; I, islets; MLC, mixed lymphocyte culture, MLIC, mixed lymphocyte-islet culture; PHA, phytohemagglutinin; x, x-irradiated lymphocytes.

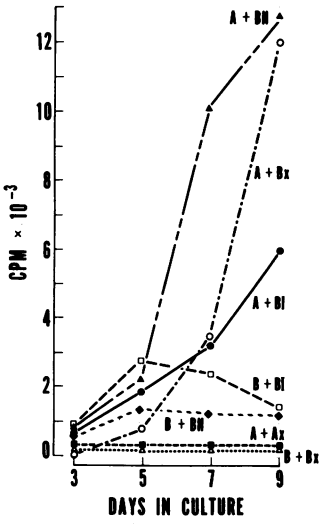

FIGURE 1 Primary MLIC reactions of MLC reactors. The kinetics of primary mixed cell cultures of lymphocytes stimulated with autologous islets (B + BI) and allogeneic islets (A + BI) are compared with the reactions of lymphocytes to autologous lymphocytes (B + Bx), allogeneic lymphocytes (A + Bx), autologous hepatocytes (B + BH), and allogeneic hepatocytes (A + BH). Mean values of [^{3}H]thymidine incorporation (counts per minute × 10^{-3}) are shown for one of five pairs of dogs responding similarly. Reactivity of B + BH was slightly higher than B + Bx but similar to BH cultured alone (not shown).

the other hand, lymphocytes dissociated from canine lymph nodes by using the collagenase method used to isolate islets, stimulated the same lymphoproliferative responses in primary MLC as did lymphocytes obtained from noncollagenase treated lymph nodes, or peripheral blood, i.e., strong allogeneic but no autologous responses. Control experiments revealed that the activity of BI in the absence of responding lymphocytes was in the range of the autologous MLC controls (B + Bx and A + Ax). Addition of fetal calf serum to these BI cultures did not affect background activity. Also, islets that had been cultured in either dog or fetal calf serum evoked similar lymphoproliferative responses, whereas medium removed from islets after 48 h in culture did not stimulate any lymphoproliferative responses.

To determine whether the lymphoproliferative response to autologous collagenase-isolated islets (B + BI; Fig. 1) might be tissue specific, since no autologous response was observed using similarly prepared lymphocytes from lymph nodes as stimulating cells, collagenase-isolated hepatocytes were also tested (Fig. 1). Isolated hepatocytes stimulated an allogeneic lymphoproliferative response (A + BH) similar to the allogeneic MLC response (A + Bx), whereas there was no detectable autologous response to either hepatocytes (B + BH) or lymphocytes (A + Ax) ($P < 0.005$).

To determine whether the observed stimulation of lymphocytes by islets (A + BI, and B + BI; Fig. 1) might have been due to contaminating passenger lymphocytes within the islets, whole islets were dissociated into single cells by using the EGTA/trypsin method (7). The cells were fixed in methanol on slides and stained with Wright-Giemsa reagent for differential counting. Less than 2% of the cells were lymphocytes. Therefore, addition of 100 islets (~200,000 cells) in MLIC would provide no more than ~4,000 lymphocytes, and this number of lymphocytes used as stimulating cells in MLC was insufficient to stimulate a primary or secondary proliferative response from 100,000 allogeneic lymphocytes. Also, x irradiation (1,500 rad) of islets did not alter their ability to stimulate allogeneic or autologous responses. Since this radiation dose completely abolishes canine MLC responsiveness, "back-stimulation" of putative lymphocytes in the BI islets by the "responding" A lymphocytes could not be responsible for [^{3}H]thymidine incorporation in the MLIC combination. Although back-stimulation has also been attributed to lymphokines elaborated from x-irradiated T cells (13), the small number of lymphocytes (~4,000) present in the islets used as stimulating cells in MLIC, when used as the only stimulating cells in MLC did not elicit detectable responses from 100,000 allogeneic lymphocytes. Conversely, x irradiation (1,500 rad) of responding lymphocytes abolished reactions to either allogeneic or autologous islets.

Secondary MLIC of MLC reactive pairs. The five pairs of animals with strong MLC reactivity in primary cultures (Fig. 1) were also studied in secondary MLC and MLIC combinations (Fig. 2). Allogeneic primed

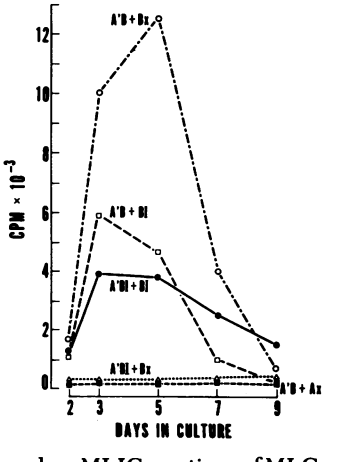

FIGURE 2 Secondary MLIC reactions of MLC reactors. Lymphocytes were primed with allogeneic lymphocytes (A'B) or islets (A'BI) for 9 d, as shown in Fig. 1, and the resultant cells were then harvested and challenged with allogeneic lymphocytes or islets. Mean values of [^{3}H]thymidine incorporation (counts per minute × 10^{-3}) are shown for one of five pairs of dogs responding similarly.

lymphocytes (A'B) were tested against the original stimulating lymphocytes in a secondary reaction (A'B + Bx), and this showed accelerated reactivity with a peak response at 3–5 d. Lymphocytes primed with allogeneic lymphocytes and tested with islets (A'B + BI) showed secondary reaction kinetics similar to A'B + Bx, but the magnitude of the response was less. Lymphocytes primed with allogeneic islets and tested with islets (A'BI + BI) also showed accelerated secondary reaction kinetics, peaking at 3–5 d. However, the response of lymphocytes primed with allogeneic islets and subsequently tested with lymphocytes autologous with the islets (A'BI + Bx) was negligible, and similar to base-line reactivity of the autologous control (A'B + Ax). Furthermore, a primary allogeneic type reaction (A + Bx) that might have been expected, even if a secondary reaction did not occur, was not observed in the A'BI + Bx cultures.

Two MLC-reactive pairs were then tested by priming lymphocytes with autologous islets for 10 d and then testing with allogeneic lymphocytes or hepatocytes (Fig. 3). Priming lymphocytes with autologous islets inhibited subsequent primary allogeneic reactivity against lymphocytes or hepatocytes (compare B'BI + Ax (AH) and B + Ax (AH)). Thus, the B'BI + Ax and B'BI + AH reactions were as low as that seen when lymphocytes were primed with autologous islets and tested with autologous lymphocytes (B'BI + Bx). In contrast, lymphocytes placed in culture for 10 d with autologous lymphocytes (B + Bx) responded strongly to allogeneic lymphocytes in a secondary culture, i.e., the B'B + Ax response was equal to the B + Ax response (not shown).

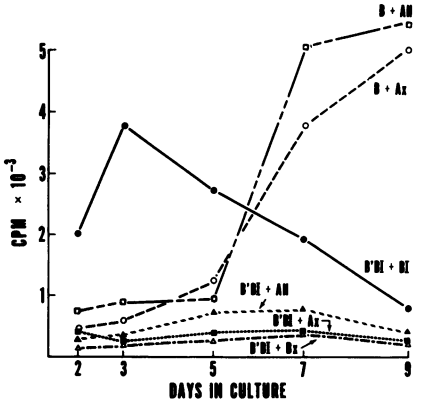

FIGURE 3 Specificity of the autologous primed MLIC. Lymphocytes of an MLC responder were primed with autologous islets for 9 d and these cells (B'BI) responded to autologous islets (B'BI + BI), and not to allogeneic lymphocytes (B'BI + Ax) or hepatocytes (B'BI + AH), whereas primary allogeneic MLC reactions (B + Ax and B + AH) were present. Mean values of [^{3}H]thymidine incorporation (counts per minute × 10^{-3}) are shown for one of two pairs of dogs responding similarly.

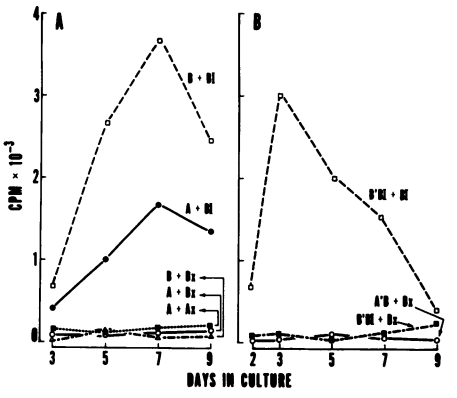

FIGURE 4 Primary and secondary MLIC reactions of MLC nonreactors. (A) The differential responses of lymphocytes to allogeneic islets (A + BI) vs. autologous islets (B + BI) in primary MLIC. (B) The kinetics and specificity of the secondary reaction of lymphocytes primed with autologous islets (B'BI) and then challenged with autologous islets (B'BI + BI) or lymphocytes (B'BI + Bx). A'BI + BI responses (not shown) were similar to B'BI + BI. Mean values of [^{3}H]thymidine incorporation (counts per minute × 10^{-3}) are shown for one of five pairs of dogs responding similarly.

MLIC of MLC nonreactive pairs. Five pairs of animals were chosen for their low MLC reactivity (nonreactors), i.e., the allogeneic reaction (A + Bx) was equal to autologous controls (A + Ax and B + Bx) although the lymphocyte responses to unrelated "indifferent" stimulator lymphocytes was pronounced. In randomly bred dogs, about one in eight will be nonreactive in MLC (in over 500 pairs studied). A nonreactor pair combination is shown in Fig. 4A, i.e., no response was observed in the allogeneic MLC (A + Bx). However, a response was elicited by autologous islets (B + BI), and the peak reaction was even greater than that seen with allogeneic islets (B + BI > A + BI at 5 and 7 d, $P < 0.01$ in five pairs). As shown in Fig. 4B, the secondary response of lymphocytes with autologous islets, to a second autologous islet stimulus (B'BI + BI), was similar in amplitude and kinetics to the secondary allogeneic MLIC reaction of a strong MLC reactor pair (Fig. 2, A'BI + BI). Again, this contrasts to the absence of reactivity seen when lymphocytes were primed against autologous islets and then tested with autologous lymphocytes (B'BI + Bx).

Effects of culturing islets on the MLIC. Since there is recent evidence that a period of tissue culture as short as one week may improve islet allograft survival (14, 15), we next examined whether a period of tissue culture would alter the ability of islets to stimulate either autologous or allogeneic lymphocyte responses in vitro. Islets isolated from a single donor pancreas were tested in MLIC after the usual one day of culture at 37°C (Methods) and again after intervals in culture ranging from 2 to 7 d at 37°C, or for 7 d at 24°C. Islet

numbers limited the study to two culture periods for each pancreas. The response in MLIC of five pairs of the MLC reactive group and two pairs of the MLC nonreactive group are shown in Table 1. There was no change in the lymphocyte stimulating capacity of either allogeneic or autologous islets which had been maintained in culture for 1–7 d at 37°C or for 7 d at 24°C. Also, islets cultured at 24° or 37°C for 7 d contained similar small numbers of lymphocytes (<2%), similar to the number present in islets cultured for only 1 d at 37°C. Similarly, lymphocytes cultured for up to 7 d at 37°C had unchanged. MLC-stimulating activities (not shown).

Glucose and insulin levels in the MLIC. Islet beta cells secrete insulin in tissue culture, and since this hormone might influence lymphocyte responses in vitro (16) glucose and insulin concentrations were measured in the cultures (Table II). In both MLC reactor and nonreactor pairs in MLIC, the concentration of glucose in the culture medium decreased from an initial value of 175 mg/dl to levels between 30 and 45 mg/dl after 9 d. These decreases were similar in the allogeneic (A + BI) and autologous (B + BI) MLIC combinations, and the glucose concentrations at 9 d in MLIC combinations (30–45 mg/dl) were significantly lower than in MLC combinations (125–130 mg/dl). Nevertheless, if glucose was added to the MLIC cultures to achieve a concentration of 100–120 mg/dl at 7–9 d, there was no change in MLIC reactivity, compared to MLIC responses observed without such additional glucose supplementation.

The concentration of insulin in the culture medium of the MLIC was between 72 and 127 ng/ml during the 9 d of culture, and this level was not significantly different in the A + BI combinations of MLC reactive or nonreactive pairs (Table II). Therefore, the dif-

TABLE I

Effects of Short-term Tissue Culture of Islets on Their Stimulating Capacity in MLIC

	Tissue culture temperature	Days of islet culture*					
		1	2	3	4	5	7
	°C	*cpm*					
MLC reactor pairs							
Pair 1	37						
A + BI		7,000‡	—§	—	8,500	—	—
B + BI		3,500	—	—	3,700	—	—
Pair 2	37						
A + BI		6,500	—	—	—	—	5,500
B + BI		4,000	—	—	—	—	4,200
Pair 3	37						
A + BI		—	8,000	—	—	6,500	—
B + BI		—	3,200	—	—	3,000	—
Pair 4	24						
A + BI		4,249	—	—	—	—	3,266
B + BI		1,369	—	—	—	—	2,834
Pair 5	24						
A + BI		5,188	—	—	—	—	4,400
B + BI		5,324	—	—	—	—	5,387
MLC nonreactor pairs							
Pair 1	37						
A + BI		—	2,000	2,300	—	—	—
B + BI		—	2,500	2,500	—	—	—
Pair 2	37						
A + BI		—	2,600	—	—	—	2,000
B + BI		—	3,000	—	—	—	2,800

* Islets were placed in tissue culture at 37°C for 1 d (see Methods for islet preparations) and further cultured for 2–7 d at 37°C or 7 d at 24°C before adding in MLIC (A + BI or B + BI).

‡ Peak [^{3}H]thymidine response in cpm; mean of triplicate cultures with <15% variance. Although only the peak response value is indicated, [^{3}H]thymidine incorporation was measured at 3, 5, 7, and 9 d in MLIC and the kinetics of reactivity were not different at either of the two time periods of islet culture studied for each pair tested. [^{3}H]thymidine values in autologous MLC combinations (A + Ax and B + Bx) was <300 cpm.

§ Not tested.

TABLE II
Glucose and Insulin Levels in MLIC and MLC

	Duration of culture				
	4 h	3 d	5 d	7 d	9 d
MLIC in MLC nonreactors*					
A + BI					
Glucose, *mg/dl*	175±2	77±20	48±12	43±5	38±5
Insulin, *ng/ml*	77±10	127±11	104±14	95±18	80±9
B + BI					
Glucose, *mg/dl*	178±2	64±10	50±9	56±8	45±6
Insulin, *ng/ml*	90±11	124±20	94±11	88±21	72±9
MLIC in MLC reactors‡					
A + BI					
Glucose, *mg/dl*	175±2	79±18	50±7	40±7	30±7
Insulin, *ng/ml*	80±5	121±34	96±27	100±24	83±10
B + BI					
Glucose, *mg/dl*	178±2	115±21	59±17	52±6	36±8
Insulin, *ng/ml*	97±22	109±21	81±22	90±28	71±8
MLC nonreactors‡					
A + Bx					
Glucose, *mg/dl*	168±2	147±3	136±5	130±3	125±11
MLC reactors‡					
A + Bx					
Glucose, *mg/dl*	160±3	150±1	145±2	133±4	130±4

* Values indicate means±SEM for four pairs of animals.
‡ Values indicate means±SEM for three pairs of animals.

ferences in lymphoproliferative responses observed between MLC reactive allogeneic (A + BI) and autologous (B + BI) combinations (Fig. 1) or between MLC nonreactive A + BI and B + BI combinations (Fig. 4A) did not appear to be related to differences in insulin levels in the culture media. Nevertheless, insulin secreted by islet beta cells may still have influenced lymphocyte proliferative responses in the MLIC. To examine this possibility directly, insulin was added to the MLC in different concentrations.

Effects of exogenous insulin on the MLC. Fig. 5 shows that, at the lowest concentrations of insulin tested (0.1 μg/ml), which was equal to that measured (72–127 ng/ml) in the MLIC media (Table II), the allogeneic MLC reactions (at 7 and 9 d) were amplified over those of controls in the absence of insulin (A + Bx), whereas an inhibitory effect on the MLC occurred only at a very high concentration of insulin (10 μg/ml). Insulin had no effect in the autologous MLC combination (A + Ax). It does not appear, therefore, that the levels of insulin present in the MLIC media were sufficient to explain the lower peak response (at 9 d) in the allogeneic MLIC (A + BI) compared to the allogeneic MLC (A + Bx), nor the earlier (3 and 5 d) responses in allogeneic (A + BI) and autologous (B + BI) MLIC compared to the allogeneic MLC (A + Bx, Fig. 1).

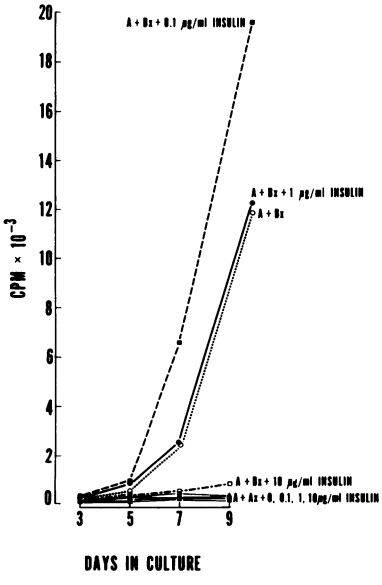

FIGURE 5 Effects of insulin (regular porcine) on the primary MLC. Stimulatory (0.1 μg/ml) and inhibitory (10 μg/ml) concentrations of insulin on the allogeneic MLC (A + Bx) are shown, whereas an intermediate (1 μg/ml) concentration of insulin had no effect. Mean values of [^{3}H]thymidine incorporation (counts per minute × 10^{-3}) are shown for one of five pairs of dogs responding similarly.

Proliferative responses of autologous lymphocytes to PHA in the presence of islets. The priming of lymphocytes with either allogeneic or autologous islets (A'BI or B'BI), leading to the exclusion of reactivity against allogeneic stimuli on lymphocytes in a secondary culture, i.e., A'BI + Bx (Fig. 2) and B'BI + Ax (Fig. 3) suggested that a suppressive effect of islets on other lymphoproliferative responses might have occurred.

To study whether islets exerted a suppressive effect on mitogen-induced lymphocyte proliferation, we tested the responses of lymphocytes to the plant lectin phytohemagglutinin (PHA) in the presence of islets. Lymphocytes (1×10^5) of five animals were cultured in 0.4% PHA with either 2×10^5 x-irradiated autologous lymphocytes (B + Bx + PHA) or 100 autologous islets (B + BI + PHA), and [^{3}H]thymidine incorporation was measured after 3 and 5 d. At 3 d of culture there was no significant difference between the mean responses in the combinations of B + BI + PHA (10,500 cpm) and B + Bx + PHA (15,600 cpm). However, at five days of culture, the mean proliferative response in the B + BI + PHA combination was significantly lower (11,320 cpm) than that observed in the B + Bx + PHA combination (32,360 cpm, $P < 0.01$). The latency in the development of suppression of mitogen-induced lymphoproliferation suggested that a suppressor lymphocyte population might have been generated as a result of the presence of islets in the culture.

Suppressive effects of lymphocytes primed against autologous islets. Since many of our observations suggested the presence of suppressor functions in lymphocytes primed against islets, such as (*a*) the lack of primary and secondary type reactions in islet-primed lymphocytes if lymphocytes were the secondary stimulus (A'BI + Bx, Fig. 2; and B'BI + Ax, Fig. 3), (*b*) dampened responses of lymphocytes to PHA in the presence of islets, and (*c*) weaker primary and secondary reactions to whole islets than to lymphocytes (Fig. 1, compare A + BI and A + Bx; and Fig. 2, compare A'BI + BI and A'B + Bx), we tested this hypothesis directly, using a three-component MLC reaction, as described (17). Lymphocytes (1×10^5) were placed in culture with 100 autologous islets for 9 d. These cells (B'BI) were then washed and added back as third components to allogeneic MLC combinations (A + Bx + B'BI and B + Ax + B'BI), with equal numbers of cells (10^5)in each component. Three pairs of animals were tested and the results in a representative pair demonstrated strong suppression of allogeneic MLC responses during co-culture with B'BI cells (Fig. 6). Addition of 0.1 μg/ml of insulin, a concentration that amplified that MLC reaction (Fig. 5), did not alter this suppressive effect of islet-primed lymphocytes on an allogeneic MLC (not shown). Also, if autologous lymphocytes primed against islets were x irradiated before co-culturing in MLC, suppression was less (by ~50%) but still significant. Finally, if lymphocytes were cultured for 9 d in the absence of any stimulatory cells and then co-cultured in an allogeneic MLC, no suppressive effect was seen.

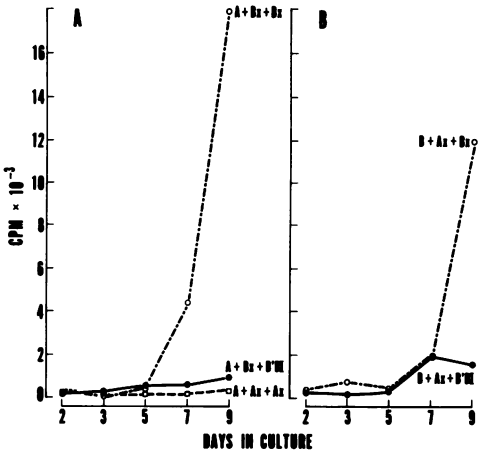

FIGURE 6 The suppressive effect of islet-primed lymphocytes on the primary MLC. Lymphocytes were primed for 9 d with autologous islets. The primed B'BI cells or Bx control cells were co-cultured as third-party cells in a primary MLC (A + Bx and B + Ax) of an MLC reactor pair. Values of [^{3}H]thymidine incorporation (counts per minute $\times 10^{-3}$) are shown for one of three pairs of dogs responding similarly.

DISCUSSION

Studies in rodents have established that isolated islets of Langerhans can reverse experimentally-induced diabetes in wholly inbred strains. However, late loss of islet function has been reported in syngeneic or weakly allogeneic settings (18, 19), and allograft acceptance has been superior using a vascularized pancreatic graft (3). Furthermore, in the human, histocompatibility matching has not prevented the rejection of transplanted islets, using standard immunusuppressive regimens (20). These observations suggested that collagenase-isolated islets might possess specific antigens amplifying alloimmune reactions after islet transplantation. In this report, we have evaluated islet immunogenicity in vitro by examining the kinetics of lymphoproliferative responses to islets in MLIC.

In experiments using pairs of animals with strong MLC responses, both allogeneic (A + BI) and autologous (B + BI) MLIC combinations were reactive (Fig. 1). However, the overall kinetics of these two MLIC reactions were different, and the peak allogeneic MLIC response (A + BI) was only half as large as that of the allogeneic MLC (A + Bx). These data indicate that the stimulating capacity of lymphocytes and islets differ, either in type or amount of lymphocyte-activating determinants. In initial studies (Methods), either one-

half or twice the amount of islets used as stimulators in MLIC did not increase the response, or alter its kinetics, suggesting that antigen concentration was not a factor. Alternatively, the differences in reactivity of A + BI and A + Bx could have reflected differences in culture media components, e.g., insulin elaborated by islets in the A + BI combination. However, additon of the concentration of insulin (0.1 μg/ml) measured to be present in an A + BI culture (Table II) increased the MLC response (Fig. 5). Also, the early reactivity in MLIC (Fig. 1; A + BI and B + BI, 3 and 5 d) was not reproduced by the addition of insulin in the MLC (Fig. 5).

The possibility that the lymphocyte response to allogeneic islets might have been due to passenger lymphocytes or Ia-containing fixed tissue macrophages must be considered, since these cells have been implicated in studies in experimental and clinical allograft rejection (21, 22). We found islets to contain less than 2% lymphocytes, so that upon addition of 100 islets (~2,000 cells/islet) a maximum of ~4,000 passenger lymphocytes might have been added per culture. This number of lymphocytes (either T or B cells) is insufficient to stimulate a primary or a secondary MLC reaction in our laboratory. Moreover, preparations of hepatic cells containing about twice as many lymphocytes as islets, and also macrophage-like Kupfer cells (in which putative Ia-like antigen might predominate) did not produce any autologous lymphoproliferative response, unlike similarly-prepared islet cells (Fig. 1). For these reasons, it is unlikely that lymphocyte and/or fixed tissue macrophages in islets caused the autostimulating effect observed (B + BI). However, the allogeneic reaction (A + BI) could perhaps have been affected by passenger lymphocytes or macrophages, acting alone or together with islet cells.

Culturing the collagenase isolated islets for up to 7 d either at 24° or 37°C did not alter their ability to stimulate autologous or allogeneic lymphocytes (Table 1). This contrasts with the reports of Lacy et al. (14, 15) of the improved survival of islet allografts in rats after a period of tissue culture, and suggests that islet-induced lymphoproliferative responses in vitro may not directly reflect islet transplantability in vivo. Perhaps a longer period of tissue culture is necessary to alter MLC reactions in dogs, as we have observed (23) in the human (4–6 wk). Alternatively, the ability to stimulate cytotoxic lymphocyte generation might be affected by shorter periods of tissue culture.

The possibility that islet specific antigens might have been unmasked or amplified by the enzymatic digestion required to isolate the islets cannot be excluded. Although collagenase treatment did not cause lymphocytes or hepatocytes to become autoimmunogenic, one might speculate that other tissues such as skin or other endocrine glands (24, 25), similarly treated, might have expressed tissue specific antigens (perhaps even cross-reacting with islets).

If islets possessed unique antigens capable of expressing immunogenicity in vitro, we reasoned that a better model than high MLC responders might be pairs of animals that did not respond in MLC. Under these conditions, any stimulation observed in MLIC would not be due to lymphocyte-defined major histocompatibility complex alloantigens exclusively. In pairs of animals without an MLC reaction, but in which there were leukocyte antigen serologically-defined disparities, autologous MLIC responses were observed (Fig. 4A), and these (B + BI) were even greater than the responses to allogeneic islets (A + BI), suggesting that weak allogeneic antigens might be inhibiting the response to specific islet antigens.

Most of the transplantation literature has dealt with recognition of alloantigens on lymphocyte targets or stimulating cells (26). Although few studies on organ or tissue specific antigens have been reported (22, 27), more recently these antigens have been invoked to explain the variability of alloimmunogenicity of different organs, e.g., kidney vs. skin (28), and as a potential cause of islet allograft rejection (3). Consequently, we questioned whether the primary and secondary MLIC possessed the typical attributes of an immunologic reaction, i.e., specificity and memory against such antigens. We found that lymphocytes responded with primary kinetics to allogeneic islets (A + BI, Fig. 1), with different kinetics of autologous islets (B + BI, Fig. 1), and with secondary kinetics to autologous islets (B'BI + BI, Fig. 3). The primary allogeneic MLIC response (A + BI, Fig. 1) resulted in cell populations (A'BI) presumably containing (*a*) cells primed against islet alloantigens shared with lymphocytes (public antigens), (*b*) cell primed against islet specific (private) antigens, and (*c*) cells with other receptors. However, the A'BI cells responded only to BI (specific antigens) and not to Bx (shared alloantigens) (Fig. 2). Similarly, in the autologous reaction, the B'BI cells responded to BI with accelerated kinetics, but did not respond to lymphocyte alloantigens (B'BI + Ax, Fig. 3).

Two possibly interrelated factors could be responsible for these observations. (*a*) the A'BI and the B'BI populations contained suppressor cells generated by islet specific as well as by allospecific responses, and (*b*) the suppressor cells were influenced by insulin. A number of reports have demonstrated that insulin receptors appear on activated T cells (29), and that insulin concentrations between 1 and 10 nM augment the cytotoxicity of allosensitive primed cells (30). Similar concentrations of insulin in our studies (0.1 μg/ml = 20 nM) enhanced MLC reactivity (Fig. 5). On the other hand, recent experiments in hypoinsulinemic mice (31), suggest that the presence of insulin is a factor

in dampening normal suppressor cell activity. Thus, in the A'BI + Bx, or B'BI + Ax combinations, since no islets (insulin) were present in the cultures, full suppressor functions might be expressed, whereas in the A'BI + BI combination, elaboration of insulin by islets (BI) would decrease the suppressor capabilities of A'BI and a positive secondary response could occur (Fig. 2). However, insulin, in a concentration that augmented primary lymphoproliferative responses (Fig. 5), did not relieve suppression (Fig. 6). This appears to exclude the possibility of suppressor mechanisms being inhibited by insulin in our experiments.

In autologous MLC, T cells respond with increased DNA synthesis to non-T cells (32–36). Sakane and Green (37) employed the bromodeoxyuridine and light technique to show that the T cells responsive to autologous non-T cells and those responsive to allogeneic non-T cells, are largely separate cell populations. These investigators also noted that the T cell population response in autologous MLC was particularly enriched in cells capable of developing suppressor capacity. Our data with lymphocytes primed with autologous islets (B'BI) are closely analogous to these findings. In direct assays for suppressor activity, the presence of autologous islets inhibited the PHA response of lymphocytes. Furthermore, the B'BI population, added as third-party cells in a fresh MLC, markedly suppressed the allogeneic MLC reaction (Fig. 6). This observation would therefore explain the failure of B'BI cells to respond to lymphocyte alloantigens in secondary MLIC (Fig. 3). However, the B'BI population responded to BI, indicating that the suppressor mechanism appeared to distinguish between alloantigens (nonself) and islet antigens (self).

Finally, it is doubtful that the observed suppressive effect of B'BI cells was due to a cytotoxic mechanism, i.e., B'BI cells cytotoxic to B responding cells or to Ax stimulating cells, thereby inhibiting the reaction (Fig. 6). Although a recent report by Tomonari (38) demonstrated autologous MLC-generated cytotoxic cells that have characteristics of natural killer cells, the major histocompatibility antigens were not the targets. Moreover, the ratio of cells employed in our studies (1:1) is much less than the ratio necessary to demonstrate autologous MLC-generated natural killer cells (~40:1).

In conclusion, this study provides evidence for both auto- and alloimmunogeneic determinants on isolated canine islets. These findings may be relevant to the increasing evidence for an association between autoimmunity, the major histocompatibility complex, and viral infections in the etiology of insulin-dependent diabetes. Humoral immunity to iselt cell antigens has been demonstrated in recent-onset, insulin-dependent diabetes in man (39, 40). Cellular immunity, although present histologically as isletitis accompanying acute-onset diabetes (41), has not been extensively measured (42). As a result, assessment of the immunoregulatory phenomenon that might determine whether the disease state will or will not emerge has been lacking. Through further studies we might begin to measure these phenomena and possibly, in time, their control.

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