

# Anti-Beta-Cell Immunity in Insulinopenic Diabetic Dogs

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## SUMMARY

Anti-islet immunity was studied in six spontaneously insulin-dependent diabetic (IDD) dogs, using mouse islets of Langerhans cells as targets, *in vitro*. Insulinopenia was demonstrated in all dogs by an i.v. glucose tolerance test. A significant lymphocytopenia was detected in the peripheral blood of this diabetic group. Pancreatic tissue from one of these animals was obtained shortly after death and the islets displayed a marked loss in beta cells without significant changes in the other types of islet cells. No insulinitis was observed.

Circulating mononuclear cells from the diabetic dogs induced an increased basal insulin (IRI) release from islet cells and a suppressed stimulated IRI release. Damage to or depth of beta cells may account for these findings.

The stimulated IRI release was also suppressed when islets were incubated with the diabetic sera + complement, while the D-cell response to arginine was not altered, and the A-cell response was reduced but not abolished. A lysis of islet cells in the presence of IDD sera + complement was demonstrated by an increased release of <sup>51</sup>Cr from labeled cells. These anomalies were observed neither when complement was heat-inactivated nor in the presence of control sera + complement.

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Several lines of evidence suggest that anti-islet immune phenomena can be involved in the destruction of insulin-secreting cells in human type I diabetes,<sup>1</sup> as well as in the spontaneous diabetes mellitus of the BB rat,<sup>2</sup> the C57 BL/KsJ-db/db mouse,<sup>3</sup> and the NOD mouse.<sup>4</sup>

In dogs, spontaneous diabetes mellitus is a common endocrine disorder.<sup>5</sup> As in human patients, this syndrome presents with a clinical diversity that may reflect a broad spec-

trum of pathophysiologic mechanisms.<sup>6</sup> In lean dogs, diabetes mellitus is associated with weight loss, polyuria-polydipsia, hyperglycemia, and ketosis.<sup>7</sup> In this variety of canine diabetes, insulinopenia<sup>8</sup> and pathologic alterations of islets<sup>9</sup> have been detected, suggesting that this syndrome may be in some way similar to human insulin-dependent diabetes (IDD). No data were available up to now to support an involvement of autoimmune phenomena in the pathophysiology of this canine IDD syndrome.

The aim of the present work is to seek cellular and humoral anti-islet autoimmunity in canine IDD, using *in vitro* functional tests, based on the alteration of beta-cell responses to stimuli in the presence of lymphocytes or sera from these dogs. Similar tests have been used previously to analyze anti-islet immunity in human IDD patients<sup>10,11</sup> and in the C57 BL/KsJ-db/db mice.<sup>3</sup> A test for the detection of cytolysis *in vitro*, based on <sup>51</sup>Cr release from labeled islet cells, was used concomitantly.

## MATERIALS AND METHODS

**Dogs.** Six IDD dogs and six healthy control dogs were obtained from private veterinarian clinics. Clinical characterization of the IDD and control dogs appears in Table 1. All animals were tested for glucose tolerance and insulin (IRI) secretion using an i.v. glucose load (500 mg/kg). Circulating white blood cells were enumerated in peripheral blood by conventional methods.

**Lymphocytes and sera.** Mononuclear cells from each dog were prepared from a 20-ml heparinized blood sample, using a Ficoll Hypaque density gradient.<sup>12</sup> The cell suspensions were adjusted to a concentration of 4.10<sup>6</sup> cells/ml in minimum essential medium (MEM Eagle, Flow Laboratories, Rockville, Maryland) supplemented with 10% fetal calf

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TABLE 1  
Clinical characterization of the dogs

Dog no.	Breed	Age (yr)	Sex	Weight (kg)	Duration of diabetes (yr)
IDD					
1	Poodle	2	F	3.8	0.3
2	Cocker spaniel	5	F	6	0.4
3	Cocker spaniel	4	M	7	1
4	Poodle	9	F	4	4
5	German shepherd	3	M	14	1.7
6	Poodle	5	F	4.5	2
Control					
1	Cocker spaniel	6	F	10	—
2	Doberman	8	F	25	—
3	Poodle	11	M	6	—
4	Beagle	1	M	12	—
5	Cocker spaniel	3	M	9	—
6	Poodle	4	F	5	—

serum, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 0.814 mg/L nonessential amino acids, 100 µg/ml streptomycin, and 100 U/ml penicillin. This medium, containing 5.5 mmol/L glucose, is hereafter termed "basal medium."

Sera were heat-inactivated (56°C for 30 min) and stored at -80°C. Pooled normal guinea pig sera, stored at -80°C, were used as a source of complement.

**Islets and islet cells.** Islets were prepared from adult mouse pancreata by the collagenase method.<sup>13</sup> Islet-cell suspensions were obtained from freshly isolated islets using EDTA and Dispase (Boehringer, Mannheim, FRG).<sup>14</sup> Before tests, islets and islet cells were incubated for 5 h in the basal medium at 37°C under air + CO<sub>2</sub> (95% + 5%).

**IRI release suppression tests.** The influence of sera + complement was studied as previously described.<sup>11</sup> Briefly, DBA/2 mouse islets were set into Falcon microtest plates (Becton-Dickinson and Co., Oxnard, California), 5 islets/well in 50 µl basal medium, with 50 µl control or IDD serum for 2 h. Complement (20 µl) was then added 3 times at 1-h intervals. After an 18-h incubation period, the islets were washed 4 times with 200 µl basal medium and successively incubated in the basal medium and in a stimulatory medium containing 16.6 mmol/L glucose + 5 mmol/L theophylline (Sigma Chemical Co., St. Louis, Missouri) for 30 min each time. Supernatants were collected before and after the 30-min test period for insulin determination.

Similar experiments were performed using MEM containing 5.5 mmol/L glucose + 20 mmol/L L + arginine HCl as a stimulus for B, A, and D islet cells. Supernatants were collected before and after the 30-min test period for hormone determinations.

The influence of mononuclear cells was studied as previously described.<sup>10</sup> Islet cells were incubated in microtest plates (5.10<sup>3</sup> cells/well in 100 µl basal medium), in the presence of mononuclear cells from control or IDD dogs (4.10<sup>5</sup> cells/well in 100 µl basal medium). After an 18-h incubation, the cells were washed and incubated for 5 min with either basal medium or stimulatory media (consisting of theophylline

line + either 16.5 or 22.0 mmol/L glucose). Supernatants were collected before and after the 5-min test period for hormone determinations.

**Islet-cell cytotoxicity assay.** A <sup>51</sup>Cr release test was performed according to the method of Dobersen et al.,<sup>15</sup> using mouse instead of rat islets. Briefly, DBA2 mouse islet cells were incubated at 37°C for 1 h with <sup>51</sup>Cr sodium chromate (10<sup>6</sup> cells + 0.1 mCi in 500 µl basal medium). After washing, cells were resuspended in microtest plates (5.10<sup>3</sup> cells/well in 100 µl basal medium) and incubated for 40 min with 15 µl serum. Then, 85 µl of a 1:8 dilution of complement was added and followed by a 30-min incubation. Aliquots of su-

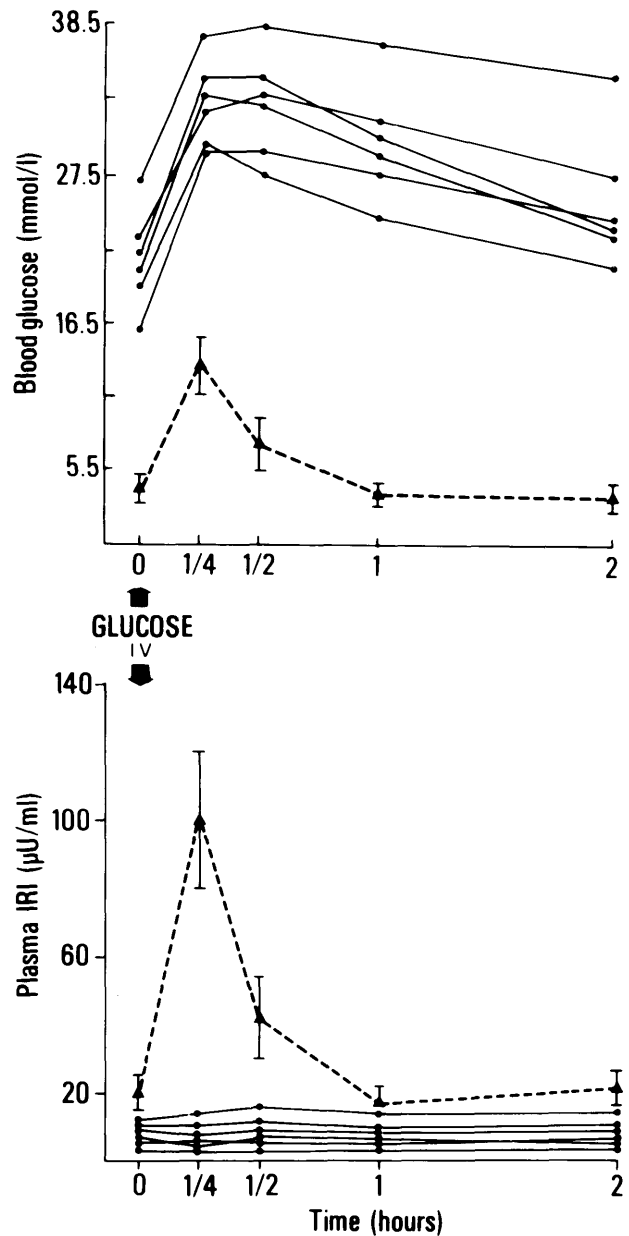


FIGURE 1. Intravenous glucose tolerance test in control (▲) or IDD (●) dogs. Serum glucose and plasma IRI values are presented, respectively, on the upper and the lower panel. Results are given as mean values ± SEM for the six control dogs and as individual values for the six IDD dogs.

pernatants were then harvested and counted in a Beckman Model 9000 gamma counter (Beckman Instruments, Fullerton, California). The percentage of  $^{51}\text{Cr}$  release, as compared with that of islet cells lysed with distilled water, was calculated.

**Morphologic study of pancreas.** The pancreas of one IDD dog (case no. 3) was obtained shortly after death and fixed in Bouin's solution. Paraffin-embedded sections were stained with hematoxylin-eosin, and for islet hormones using the peroxidase-antiperoxidase (PAP) method of Sternberger<sup>16</sup> with specific sera against insulin, glucagon, somatostatin, and pancreatic polypeptide (PP). Pancreatic sections from control dogs were similarly prepared and analyzed.

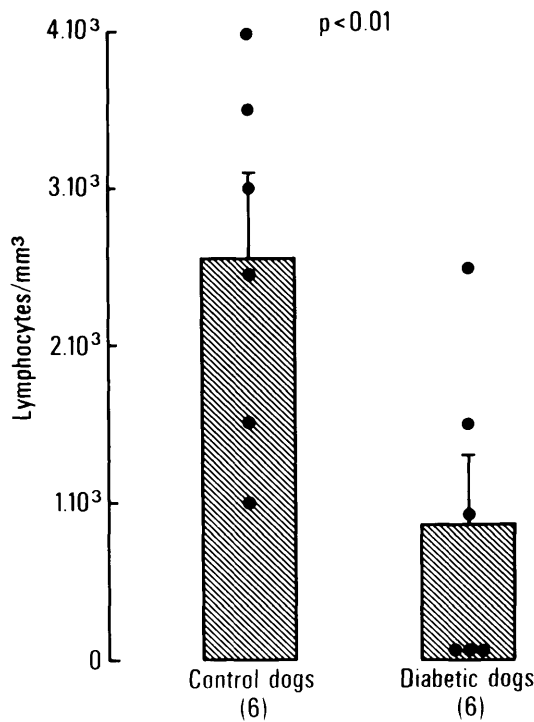
**Determinations and presentation of results.** Serum glucose concentrations were measured by the glucose-oxidase procedure. Ketonuria was assessed using Ketostix R strips (Ames Company, Elkhart, Indiana). Plasma samples and aliquots of the in vitro incubation media were frozen at  $-20^{\circ}\text{C}$  until assayed for insulin,<sup>17</sup> glucagon (IRG),<sup>18</sup> and somatostatin.<sup>19</sup> Results are presented as mean values  $\pm$  SEM, and statistical significances of differences were calculated by the Student's *t* test.

**RESULTS**

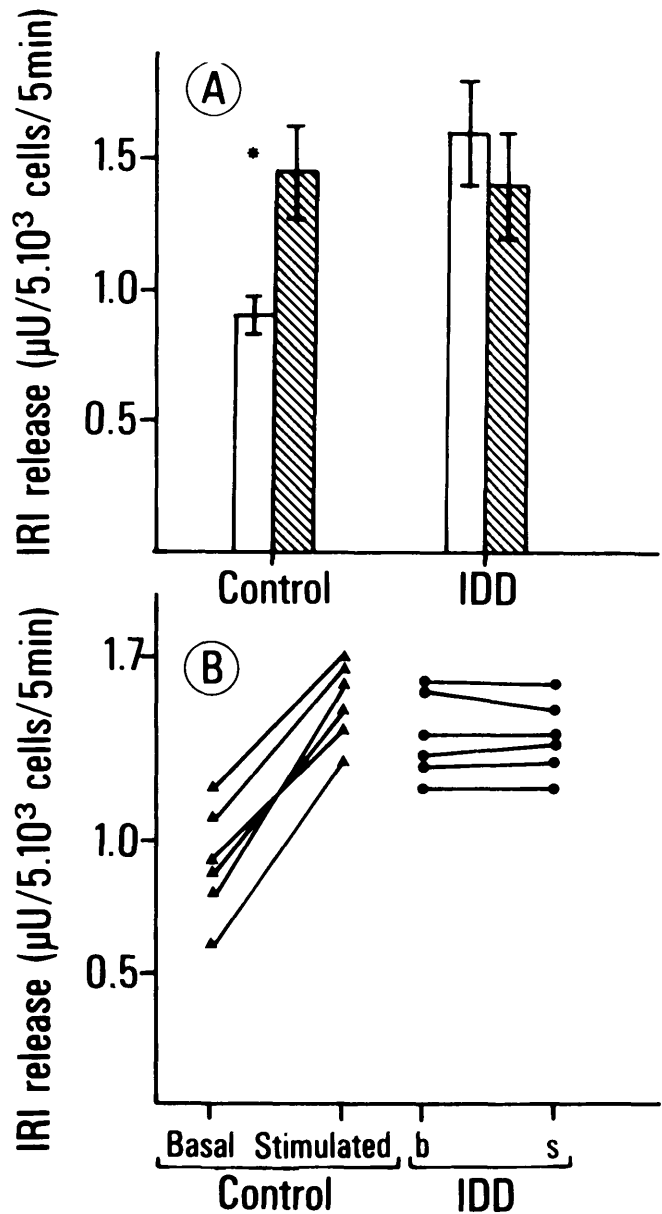
**Intravenous glucose tolerance test.** Fasting blood glucose was higher in the diabetic than in the control dogs:  $20.3 \pm 2.4$  mmol/L versus  $4.2 \pm 1.3$  mmol/L ( $N = 6$ ;  $P < 0.001$ ). Fasting plasma insulin concentration was lower in the diabetics than in controls:  $7.3 \pm 2.0$   $\mu\text{U}/\text{ml}$  versus  $20.1 \pm 6.2$   $\mu\text{U}/\text{ml}$  ( $P < 0.001$ ). After the i.v. glucose load, glucose values

were significantly higher in the diabetic dogs than in controls and no rise in plasma insulin concentration occurred, contrasting with the beta-cell response in control dogs (Figure 1).

**Peripheral white blood cell counts.** Leukocyte counts in diabetic dogs were characterized by (1) an increase in mean total white-cell number;  $24,600 \pm 4330$  cells/ $\text{mm}^3$  versus  $12,083 \pm 1920$  in control dogs ( $P < 0.01$ ); (2) an increase in neutrophil polymorphonuclear cells:  $19,600 \pm 4600$  cells/ $\text{mm}^3$  versus  $7500 \pm 1560$  ( $P < 0.01$ ); and (3) an absolute



**FIGURE 2.** Lymphocyte counts in peripheral blood from six control and six diabetic dogs. The black circles denote individual values and the hatched columns denote mean values  $\pm$  SEM. The statistical difference between control and IDD groups is indicated ( $P < 0.01$ ).

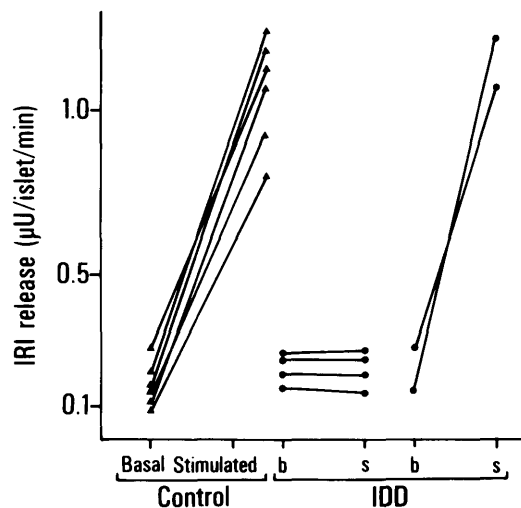


**FIGURE 3.** Insulin release from mouse islet cells incubated in vitro with mononuclear cells (MNC) from six control or IDD dogs. (A) Mean values  $\pm$  SEM. Open bars represent basal IRI releases (5.5 mmol/L glucose) and black bars, stimulated IRI releases (16.5 mmol/L glucose + 5 mmol/L theophylline). The asterisk denotes a statistical difference between the corresponding basal and stimulated releases ( $P < 0.05$ ). (B) The individual results. Each point indicates basal (b) or stimulated (s) IRI release in the presence of MNC from one control ( $\blacktriangle$ ) or IDD ( $\bullet$ ) dog. Each line joins the corresponding basal and stimulated releases.

decrease in lymphocyte number:  $850 \pm 446$  cells/mm<sup>3</sup> versus  $2580 \pm 520$  in controls ( $P < 0.05$ ). This lymphocytopenia was particularly pronounced in dogs nos. 1, 2, and 3, reaching a value less than 1/20 of the mean control value (Figure 2). These three dogs had the shortest known duration of diabetes (Table 1).

**Functional impairment of beta cells in vitro by diabetic lymphocytes.** Mouse islet cells responded significantly to the stimulation by 16.5 mmol/L glucose + theophylline:  $2.1 \pm 0.3 \mu\text{U}/5.10^3$  cells/5 min versus  $1.2 \pm 0.1 \mu\text{U}/5.10^3$  cells/5 min in the basal medium ( $N = 6$ ;  $P < 0.01$ ). Similar figures (Figure 3A) were obtained when mononuclear cells from control dogs were incubated with the islet cells:  $1.5 \pm 0.2 \mu\text{U}/5.10^3$  cells/5 min versus  $0.9 \pm 0.1 \mu\text{U}/5.10^3$  cells/5 min ( $P < 0.01$ ). This response was not maximal, and a higher IRI release was obtained in the presence of 20.0 mmol/L glucose + theophylline:  $1.9 \pm 0.2 \mu\text{U}/5.10^3$  cells/5 min (Table 2). In contrast, the presence of diabetic mononuclear cells deeply altered the beta-cell function (Figure 3A). On one hand, the IRI release in basal medium was higher than in control experiments:  $1.6 \pm 0.2 \mu\text{U}/5.10^3$  cells/5 min ( $N = 6$ ;  $P < 0.01$ ). On the other hand, no significant beta-cell response to stimulation by 16.5 mmol/L glucose + theophylline was detected:  $1.4 \pm 0.2 \mu\text{U}/5.10^3$  cells/5 min (NS versus basal value). Furthermore, at variance from controls, no supplementary IRI release occurred in the presence of 22.0 mmol/L glucose + theophylline:  $1.5 \pm 0.2 \mu\text{U}/5.10^3$  cells/5 min (Table 2). Mononuclear cells from each of the six IDD dogs altered the beta-cell function in vitro (Figure 3B).

**Suppression of IRI release in vitro by IDD sera + complement.** In the absence of canine sera + complement, IRI release from islets was  $0.3 \pm 0.1 \mu\text{U}/\text{islet}/\text{min}$  in the basal medium; it was significantly increased by addition of 16.5 mmol/L glucose + 5 mmol/L theophylline:  $1.8 \pm 0.3 \mu\text{U}/\text{islet}/\text{min}$  ( $N = 6$ ;  $P < 0.001$ ). In the presence of control sera + complement, the stimulated IRI release was, similarly, higher than basal values ( $P < 0.001$ ), as shown in Figure 4. Four of the six IDD sera suppressed the beta-cell response to glucose + theophylline in the presence of complement, while the insulin release in basal medium was in the control range (dog nos. 1–4). In the presence of heat-inactivated complement, these four IDD sera did not suppress the beta-cell response to the stimulation:  $0.2 \pm 0.2 \mu\text{U}/\text{islet}/\text{min}$  in the basal medium versus  $1.4 \pm 0.4 \mu\text{U}/\text{islet}/\text{min}$  in the stimulatory medium ( $N = 4$ ;  $P < 0.001$ ). With the two other IDD sera (dog nos. 5, 6), neither the basal nor the stimulated IRI secretions were altered in the presence of complement (Figure 4). When complement was omitted, none of the IDD



**FIGURE 4.** Insulin release from islets incubated in vitro in the presence of the six individual control (▲) or IDD (●) sera, plus complement. The four IDD sera that alter IRI release are presented on the middle panel. The two IDD sera that do not modify IRI release are shown on the right panel. The presentation is the same as in Figure 3.

sera suppressed the beta-cell response to glucose + theophylline.

A significant increase in IRI, IRG, and somatostatin releases occurred when islets were stimulated by L + arginine in the absence of any canine serum, or in the presence of control sera + complement. Addition of IDD sera + complement reduced significantly the basal IRI release and suppressed the beta-cell response to arginine, while neither the basal somatostatin release nor the D-cell response to arginine was altered (Table 3). During the same experiments, the basal IRG release was increased and the A-cell response to arginine was decreased but remained significantly higher ( $P < 0.01$ ) than the basal value.

**<sup>51</sup>Chromium release from islet cells.** When <sup>51</sup>Cr-labeled islet cells were incubated with complement in the absence of canine sera, the <sup>51</sup>Cr release was  $39 \pm 3\%$  ( $N = 6$ ). In the presence of control sera + complement, the amount of <sup>51</sup>Cr released was  $42 \pm 3\%$  ( $N = 6$ ). In contrast, treatment of cells by sera from the six IDD dogs + complement resulted in a significantly increased <sup>51</sup>Cr release (Figure 5). When the same IDD sera were incubated in the presence of heat-inactivated complement, the <sup>51</sup>Cr release from cells was not different from that obtained with control sera + complement:  $45 \pm 4\%$  versus  $42 \pm 3\%$ , respectively ( $N = 6$ ).

**TABLE 2**  
Effect of mononuclear cells (MNC) on insulin release from mouse islets

	Glucose (mM)	Theophylline (mM)	Insulin release without MNC	Control (MNC) ( $\mu\text{U}/5.10^3$ cells/5 min)	IDD (MNC)
A (6)	5.5	0	$1.2 \pm 0.1$	$0.9 \pm 0.1$	$1.6 \pm 0.2\text{\$}$
B (6)	16.5	5	$2.1 \pm 0.3^*$	$1.5 \pm 0.2\ddagger$	$1.4 \pm 0.2$
C (4)	22.0	5	Not tested	$1.9 \pm 0.2\ddagger$	$1.5 \pm 0.2\parallel$

Results are presented as mean values  $\pm$  SEM for the number of experiments indicated in parentheses. Statistical significances of differences are indicated as follows: A versus B: \* $P < 0.01$ , † $P < 0.01$ ; B versus C: ‡ $P < 0.05$ ; control versus IDD: § $P < 0.01$  and || $P < 0.05$ .

TABLE 3

Insulin, glucagon, and somatostatin secretions from mouse islets incubated in the presence of control and diabetic sera + complement, then submitted to a stimulation by L (+) arginine (20 mmol/L)

	Insulin ( $\mu$ U/islet/min)		Glucagon (pg/islet/min)		Somatostatin (ng/islet/min)	
	Basal	Stimulated	Basal	Stimulated	Basal	Stimulated
Control sera (3)	0.08 $\pm$ 0.01	0.23 $\pm$ 0.01	0.07 $\pm$ 0.01	0.75 $\pm$ 0.01	0.03 $\pm$ 0.01	0.61 $\pm$ 0.02
Diabetic sera (3)	0.05 $\pm$ 0.01*	0.05 $\pm$ 0.01‡	0.20 $\pm$ 0.01†	0.35 $\pm$ 0.01†	0.03 $\pm$ 0.01	0.58 $\pm$ 0.02

Results are presented as mean values  $\pm$  SEM. Number of sera tested appears in parentheses. \*P < 0.05; †P < 0.01; ‡P < 0.001.

**Islet pathology.** On diabetic dog (no. 3) pancreatic sections stained with hematoxylin-eosin, the islets of Langerhans were small and reduced in number. Some of the islet cells were hydropic, and the tubular cells displayed the same aspect. No insulinitis was observed. On the sections prepared for immunoperoxidase study, the insulin-positive cells were markedly reduced in number and enlarged. Some of them were degranulated, while others still contained insulin-positive granules. The glucagon-positive cells were numerous and strongly reactive for the glucagon antiserum. Many islets contained somatostatin-positive cells. The PP-antiserum revealed numerous isolated PP-cells disseminated among the exocrine tissue, as in normal dog pancreas. Compared with the pancreas of control dogs, the pancreas of this IDD dog thus displayed a loss of beta cells with hypertrophy of the remaining beta cells and pseudohyperplasia of the A-cells.

## DISCUSSION

The present study suggests that in some insulinopenic diabetic dogs, circulating mononuclear cells, on the one hand, and sera + complement, on the other, can alter the insulin release from mouse islet cells in vitro. Islet cell lysis induced by sera + complement was also suggested from  $^{51}\text{Cr}$  release tests. In one dog whose pathology could be studied, the islets of Langerhans were scarce and the beta cells were reduced in number, while no abnormality was observed in the other islet cells.

Diabetes mellitus in dogs is not a rare disease, and its frequency is estimated at 1/200.<sup>5</sup> Detailed clinical, biochemical, and pathologic characterization was difficult because of obvious psychological reasons. However, a detailed pathologic study has been published,<sup>9</sup> which demonstrates that,

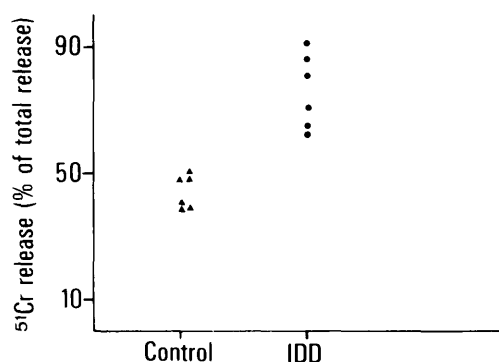


FIGURE 5. Chromium release from  $^{51}\text{Cr}$ -labeled islet cells, in the presence of the six control (▲) or IDD (●) sera. Results are given as percent of the total Cr release from distilled water-lysed cells.

in pancreata from 30 diabetic dogs, islets were scarce and beta cells were reduced in number. This morphologic observation is consistent with the low-plasma IRI concentrations, which were measured in a few instances.<sup>8,20</sup> Insulinopenia was found in approximately 50% of the cases studied. In the present series, all six dogs were severely insulinopenic, as demonstrated by an i.v. glucose tolerance test.

Some anomalies in white-blood-cell counts were also detectable in all six IDD dogs. They look consistent with the few data available in the literature,<sup>21</sup> and are similar to the profound lymphocyte anomalies recently documented in the BB rat,<sup>22,23</sup> the C57 BL/KsJ-db/db mouse,<sup>24</sup> and some IDD patients.<sup>25-28</sup> The increase in polymorphonuclear cells was presumably related to the pyrogen infections of skin and urinary tract present in these dogs. This is the first report of anti-islet immunity in canine IDD. Anti-islet complement-dependent humoral immunity was demonstrated by an impairment of beta-cell function and by a lysis of islet cells. This humoral immune process appeared mainly directed toward the beta cells, as the somatostatin release from D-cells was not modified. An alteration of IRG release was detectable and may indicate a lysis of A-cells that did not reach a complete suppression of the stimulated hormonal release as in the case of IRI secretion. Such an aggression of A-cells was not detectable in our previous studies with sera from human IDD<sup>11</sup> or from C57 BL/KsJ-db/db diabetic mice.<sup>3</sup> It may be specific of canine IDD, but would be inconsistent with the selective beta-cell damage occurring in canine as in human IDD.<sup>9</sup> The significance of this phenomena thus requires further investigation. On the other hand, as compared with our studies with human IDD sera,<sup>11</sup> it can be noted that canine IDD sera + complement did not lead to an increased basal IRI release. This apparent discrepancy between the two systems may be due mostly to the extensive washing of islets after the preliminary incubation in the present report. These washings may eliminate the passive IRI leakage from dead cells, which is the explanation for the increased basal IRI release supported by our<sup>11</sup> and other<sup>29</sup> reports.

Anti-beta-cell cellular immunity was suggested by a suppressed stimulated IRI release and an increased basal release. The higher-than-normal basal IRI release in the presence of canine IDD mononuclear cells was not noted, using a similar test, with human IDD lymphocytes.<sup>10</sup> It may result either from a stimulatory effect of canine IDD lymphocytes on IRI secretion at 5.5 mmol/L glucose or from a passive insulin leakage from damaged beta cells.

The latter hypothesis appears the most likely, although the viability of islet cells could not be assessed in this system.

Consistent with this possibility, a significant lysis of RINm-5F rat insulinoma cells was induced by lymphocytes from one IDD dog, as demonstrated by the eosin exclusion test (20% of dead cells versus 7% in the presence of lymphocytes from one healthy dog). However, a stimulatory effect of canine IDD lymphocytes on basal IRI release cannot be excluded from this study.

Nevertheless, the high basal IRI release in the presence of IDD lymphocytes did not account for the block in IRI secretion stimulated by 16.5 mmol/L glucose + theophylline, since this stimulus was not maximal in the present system: a supplementary IRI release occurred during stimulation by 20.0 mmol/L glucose + theophylline in the presence of control, but not of IDD, lymphocytes.

Although the discrepancies with findings concerning human IDD require enlightenment, the insulin-dependent diabetic dog represents a new animal model in which anti-islet immunity is involved.

#### ACKNOWLEDGMENTS

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