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^{111}Indium-labelled lymphocytes do not image or label the pancreas of the BB/W rat

D. L. Gallina[1], D. Pelletier[1], P. Doherty[1], S. B. Koevary[1], R. M. Williams[2], A. A. Like[1], W. L. Chick[1] and A. A. Rossini[1]

[1]University of Massachusetts Medical School, Worcester, Massachusetts, and [2]Northwestern University School of Medicine, Chicago, Illinois, USA

Summary. Autologous transfusions of ^{111}indium-labelled peripheral blood lymphocytes reportedly image the pancreas of patients with Type 1 (insulin-dependent) diabetes at the time of onset. We attempted to apply this technique to the spontaneously diabetic BB/W rat. First, acutely diabetic BB/W rats, diabetes-prone BB/W rats, diabetes-resistant W-line BB/W rats, and Wistar Furth rats were given autologous transfusions of labelled peripheral blood lymphocytes. Radioactivity recovered from the pancreas was similar in all groups. No correlation was found between the intensity of imaging and the presence or intensity of insulitis. To decrease non-specific intravascular radioactivity, acutely diabetic, diabetes-prone, and W-line rats were perfused 48 h after autologous transfusion of labelled lymphocytes. Again, the intensity of recovered activity was similar in all groups, using both macroautoradiography and numerical counting techniques. A second set of experiments studied diabetes and insulitis induced by passive transfer of concanavalin A-treated splenic lymphocytes from acutely diabetic donors. Activated lymphocytes were labelled with ^{111}Indium and given to groups of diabetes-prone and diabetes-resistant rats. There were no differences in pancreatic localization 72–96 h after injection. Groups of diabetes-prone and diabetes-resistant rats were also given concanavalin A-activated lymphocytes and then challenged 2–10 days later with autologous transfusions of labelled peripheral blood lymphocytes. Again, no differences in organ labelling or imaging were detected. We conclude that the autologous transfusions of ^{111}indium-labelled lymphocytes do not label or image the pancreas of the BB/W rat.

Key words: Insulin-dependent diabetes, autoimmunity, BB rat, insulitis, ^{111}Indium labelled lymphocytes, pancreatic imaging.

Considerable evidence suggests that human Type 1 diabetes is an autoimmune disease [1]. A convincing observation in support of this hypothesis is the lymphocytic infiltration of the islets of Langerhans (insulitis) found in patients at the time of onset [2]. Pancreatic tissue is not readily obtained except at post mortem examination, however, and it would be desirable to develop a non-invasive method to detect and quantify insulitis.

Recently, an imaging technique has been reported to define and trace the course of inflammatory processes using radionuclides [3]. Peripheral white blood cells are isolated, tagged with ^{111}indium, and re-injected into the circulation. The migration of these cells to inflamed tissues is then documented with images obtained by standard scanning procedures [4–6]. A recent report describes the application of this technique to three patients with newly diagnosed Type 1 diabetes [7]. Using emission computerised scanning, two of the three patients given autologous radiolabelled lymphocytes appeared to show localization of the activity in the region of the pancreas. This study suggested that the active process of islet inflammation in Type 1 diabetes could be detected non-invasively.

The BB rat develops a syndrome of spontaneous diabetes which shares many characteristics of human insulin-dependent diabetes [8]. Acutely diabetic rats have intense insulitis [9]. This study describes experiments designed to follow the migration of ^{111}indium-labelled lymphocytes to the islets of BB rats. The phenomenon was studied in spontaneously diabetic rats and in rats with diabetes and insulitis induced by the passive transfer of lymphocytes from acutely diabetic donors [10].

Materials and methods

Animals

All experiments used BB rats (designated BB/W) from the colony maintained at the University of Massachusetts Medical School, Worcester, Mass. The frequency of diabetes in these BB/W rats averages 40–60%. Acutely diabetic rats were used within 24 h of detection. Chronically diabetic rats were treated with insulin for approximately 60 days before use. Normoglycaemic diabetes-prone BB/W rats were either aged 80–95 days (the age when the incidence of diabetes is maximal) or aged 30–40 days (when the age frequency of diabetes is $< 0.5\%$).

Non-diabetic control rats were of two kinds. The first was a line of BB/W rats bred for resistance to diabetes. Only 19 of these W-line rats have become diabetic in over 18 generations of brother-sister mating ($n > 1500$). Non-diabetic Wistar-Furth rats (Microbiological Associates, Baltimore, Maryland) have the same RT1^u major histocompatibility haplotype as the BB/W rat and were also used as controls.

Peripheral blood lymphocyte isolation

Whole blood (2–4 ml) was obtained from the orbital sinus of donor rats using heparinized capillary tubes and was transferred into plastic tubes containing 7,500 units of heparin and 15 ml RPMI-1640 medium (M. A. Bio-Products, Walkers-ville, Maryland). After adjusting the volume to 20 ml with RPMI-1640, 5 ml of cell suspension were gently layered onto an equal volume of lympholyte M (Cedarline Laboratories, Hornby, Ontario, Canada) in each of four tubes. Samples were then centrifuged at 500 *g* for 20 min at 20 °C, and the lymphocytes re-

covered from the interface. The cells were washed three times with the same medium and counted with a haemocytometer (American Optical, Buffalo, New York). Viability was evaluated by the method of trypan blue exclusion and exceeded 90% in all experiments.

Labelling of lymphocytes

Lymphocytes were labelled by a slight modification of the method of Wagstaff et al. [11]. ^{111}Indium oxine (Amersham International, Arlington Heights, Illinois) was added to a 2 ml suspension of the cells at a concentration of 20–100 μCi/10^8 cells. The cells were incubated with label at room temperature for 15 min and centrifuged at 300 *g* for 13 min. After a rapid wash in RPMI-1640, the cells were resuspended in 1 ml of the same medium and immediately injected intravenously using a plastic syringe. Activity was measured in both the supernatant washes and in the full syringe to calculate labelling efficiency. After injection, the residual activity in the empty syringe was measured and the actual administered dose was calculated. Efficiency of labelling was > 80%.

Transfer of diabetes and insulitis by means of activated lymphocytes

Splenic lymphocytes from acutely diabetic BB/W rats were cultured according to the method of Koevary et al. [10]. Briefly, $100–300 \times 10^6$ cells were incubated in 150 ml RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2-mercaptoethanol (2×10^{-5} mol/l), glutamine (2 mol/l) and concanavalin A (con A) (5 μg/ml) (Miles-Yeda, Rehovot, Israel). Incubations were carried out in 150 ml flasks (Costar, Cambridge, Mass.) at 37 °C in a humidified environment with 5% CO^2 for 72 h. At the end of the incubation period, cells were harvested by gently pouring into a large conical flask. After a 10-min centrifugation at 300 *g*, the pellet was washed twice with serum-free RPMI. Cells were counted in a Coulter counter (Coulter, Edison, New York) and tested for viability. In general, the number of cells harvested ranged from 20% lower to 30% higher than the initial count. The activated cells were then injected intravenously either immediately without further modification, or after labelling with ^{111}indium oxine.

Methods of sacrifice

One group of anaesthetized animals was perfused to remove as much blood as possible from the body. A 16-gauge needle was inserted into the left ventricle and a small incision made in the right atrium. Ringer's lactate buffer (120 ml), containing 1000 units of heparin and 0.1% procaine, was then injected, followed immediately by 10% formalin for fixation. All other groups of animals were killed with CO^2 and not perfused.

Organ examination

Pancreas, thyroid, and spleen were removed immediately after sacrifice, weighed and placed into a scintillation counter (Searle, Elk Grove Village, Illinois) to measure incorporation of isotope into the organs. In some experiments, additional organs were examined (liver, kidney, lung, salivary gland and thymus). After counting, unfixed organs were immersed in Bouin's solution and later prepared for histological examination. Haematoxylin and eosin stained sections were examined by a pathologist (AAL), who was unaware of the treatment status of the rats.

Radiolabel detection methods

Gamma camera imaging. To scan the pancreas, left anterior oblique views (45°) of the upper abdomen were taken in animals anaesthetized with chloral hydrate. A gamma camera (Ohio Nuclear 420, Cleveland, Ohio) with a pinhole collimeter was employed for counting the 173 KeV energy peak of ^{111}indium. Data acquisition was for 80 K on a 128 × 128 matrix. The period of acquisition varied from 15 to 25 min depending on dose and time post-injection. All data were recorded on magnetic tape and processed with a PDP 11/24 computer. The final enhanced images were stored on Nuclear Medicine Clear Film (Kodak, Rochester, New York). The examination of the scans was performed by a nuclear medicine physician (PD), who was unaware of the treatment status of the animals.

Recovery of activity from individual organs. Individual organs were placed in plastic tubes after a rapid wash in saline solution (0.154 mmol/l) and were counted in a scintillation counter. An appropriate aliquot of ^{111}indium-oxine was counted several times throughout the run in order to assess the efficiency of the machine. The total activity found in each organ was adjusted for decay and expressed as percentage of the administered dose per g of tissue.

Macroautoradiography. Organs were embedded in Tissue-Tek II (Miles Laboratories, Naperville, Illinois) on a microtome plate and rapidly frozen in a cryostat (American Optical, Buffalo, New York). Frozen sections (1–2 mm thick) were placed on small cards, covered with plastic wrap, and placed in contact with X-ray film (X-OMAT-XAR-2, Kodak) for 48–72 h. Autoradiographs were developed using standard procedures.

Statistical analysis

All parametric data are presented as mean ± SD. Means of three or more groups were compared using one-way analyses of variance. Comparisons of two means used either the two tailed unpaired t-test or the Tukey procedure for multiple comparisons. Correlations were calculated using the Pearson product moment method [12].

Results

Fate of injected labelled peripheral blood lymphocytes in the circulation

Blood samples (2 ml) were obtained from two groups of three diabetes-prone animals aged 87 ± 4 days. Peripheral blood lymphocytes (PBL) were isolated, counted, labelled and reinjected at a dose of 45.7 ± 11.2 (group A) or 86.2 ± 16.7 (group B) μCi/10^8 cells. The persistence of labelled PBL in the circulation was assessed by serial blood sampling 24, 48 and 72 h after injection. Lymphocytes were isolated from a known volume of blood, counted, and the ^{111}indium activity measured and corrected for decay to disintegrations per minute (dpm). The percentage of labelled PBL remaining at each time was then calculated using the following formula:

$$\frac{\frac{(\mathrm{dpm})_T}{\mathrm{ml}} \times \frac{54\ \mathrm{ml}}{\mathrm{kg}} \times \frac{\mathrm{kg\ body\ weight}}{\mathrm{rat}} \times \frac{(\mathrm{PBL})T_0}{\mathrm{dpm}}}{\frac{(\mathrm{PBL})T_0}{\mathrm{rat}}} \times 100$$

Where T = 24, 48 or 72 h and T_0 = time of the injection. For this calculation it was assumed that the amount of isotope bound to each cell remained constant [13], and that the blood volume of the rat is 54 ml/kg [14]. In group A 2.69 ± 1.9, 0.25 ± 0.06 and 0.13 ± 0.04% of labelled PBL remained in the circulation 24, 48 and 72 h after injection. For group B, comparable results were 1.04 ± 0.64, 0.18 ± 0.02 and 0.10 ± 0.05%. As expected, the recovery of labelled PBL was higher in group A (low dose label) than in group B (high dose) 24 h after the injection. The results for both groups at 48 and 72 h

Table 1. Incorporation of ^{111}Indium-labelled lymphocytes into various tissues at 48 h after injection

	Percentage of administered dose recovered/g tissue			
	Acutely diabetic BB/W rats ($n=7$)	Wistar Furth rats ($n=7$)	Diabetes-prone BB/W rats ($n=9$)	W-subline rats ($n=3$)
Spleen	12.8 ±14.1	15.1 ±9.5	16.3 ±12.0	13.8 ±9.2
Lung	0.47± 0.19	0.81±0.46	–	–
Liver	1.9 ± 0.89	2.9 ±2.9	–	–
Pancreas	0.21± 0.05	0.24±0.07	0.15± 0.03	0.19±0.01
Kidney	1.7 ± 0.64	1.2 ±0.20	–	–
Thyroid	0.59± 0.21*	0.36±0.04	0.36± 0.07	0.42±0.04
Salivary	0.24± 0.06	0.29±0.18	–	–

Results expressed as mean ± SD.
*$p<0.01$ compared with Wistar Furth and diabetes-prone BB/W rats

Table 2. Incorporation of ^{111}Indium labelled lymphocytes in various organs of animals perfused 48 h after injection

	Percentage of administered dose/g of tissue		
	Acutely diabetic BB/W rats ($n=4$)	Diabetes prone BB/W rats ($n=9$)	W-subline rats ($n=4$)
Spleen	6.50±1.89	7.09±1.18	8.2 ±4.67
Pancreas	0.05±0.01[a]	0.12±0.03	0.13±0.08
Thyroid	0.18±0.03[b]	0.27±0.09	0.23±0.04

Results expressed as mean ± SD.
[a] $p<0.02$ compared with diabetes-prone BB/W and W-subline rats;
[b] $p<0.05$ compared with diabetes-prone rats

showed no significant difference. About 0.20% of the original injected lymphocytes remained in the circulation at 48 h. This figure is comparable with data reported previously [15].

Incorporation of labelled lymphocytes into various organs

Acutely diabetic and diabetes-prone animals. Four groups of age-matched animals 80–90 days were examined: acutely diabetic BB/W rats, diabetes-prone BB/W rats, W-line diabetes resistant BB/W rats, and Wistar Furth rats. PBL were separated and incubated with ^{111}indium. Animals were sacrified 48 h after the injection of the labelled cells (48.3–152 μCi/10^8 cells) and various organs were examined for incorporation of the isotope (Table 1). No significant differences were found in the radioactivity of organs among the four groups of animals, with the exception of the thyroid gland in the acutely diabetic BB/W rats. The isotopic activity was greater in this group when compared with the Wistar Furth and diabetes-prone BB/W groups. Histological examination of the pancreas was performed on the diabetes-prone and W-line BB/W rats; three out of nine and zero out of three animals in each group demonstrated insulitis. No histological data were obtained from the acutely diabetic group. However, previous studies have demonstrated that most islets have insulitis or are already at end-stage when the animals become acutely diabetic [9]. A similar study was performed in six acute diabetic and three Wistar-Furth animals, but after auto-injection of the ^{111}indium labelled spleen lymphocytes (data not shown). As for the case of PBL, no significant differences in the activity recovered from the organs was observed between the two groups.

Chronically diabetic animals. Our first set of experiments suggested an increase in the labelling of the thyroid gland in the acutely diabetic rats compared with two of the other groups of animals. Because previous data suggested that the prevalence of thyroiditis in BB rats increases with age [16], we decided to study older rats. Chronically diabetic animals ($n=15$), approximately 2 months after the onset of diabetes, and age-matched W-subline animals ($n=13$) received auto-injections of a similar number (60.2 ± 0.4 μCi/10^8 cells) of labelled PBL. Tissues were examined 48 h following injections. There were no significant differences between the two groups of animals in any non-endocrine organ examined. Recovery of label in the pancreas of the chronically diabetic BB/W was 0.22 ± 0.06 versus 0.24 ± 0.05%/g in the W-line group (NS). Thyroid recovery was 0.42 ± 0.10%/g in the chronically diabetic rats and 0.5 ± 0.17%/g in the W-line rats (NS). Histological examination revealed thyroiditis in 9 of the 15 chronically diabetic rats and only one of 13 W-line animals. It should be noted that mild thyroiditis is infrequently observed in W-line animals [16]. There was no correlation of the percentage isotopic activity recovered from the thyroids and the presence or intensity of thyroiditis.

Acutely diabetic and diabetes-prone animals perfused before examination. Since entrapment of circulating lymphocytes within the vascular system may obscure the detection of lymphocytes that have attached to target organs, several animals were perfused before organ removal. Three groups of age-matched acutely diabetic, diabetes-prone, and W-line animals were studied, using the same organ study protocol as outlined above with the addition of a perfusion step. Before organ removal, each animal was perfused with buffer and then formalin to wash out intravascular label. The migration of labelled lymphocytes was then assessed by the recovery of activity and the autoradiographic examination of the organs 48 h after the injection of 61–79 μCi/10^8 PBL.

The percentage of the administered activity recovered per g of organ is shown in Table 2. The spleen counts in all three groups were statistically similar. As expected, the total number of counts recovered from the spleens was approximately half that previously obtained from non-perfused animals (Table 1). There was, however, significantly lower thyroidal and pancreatic isotopic activity in the acutely diabetic animals compared with either the diabetes-prone or W-line groups. There were no significant differences in the activity recovered from the organs of the acutely diabetic rats, the two of the nine diabetes-prone animals with insulitis, or the animals without insulitis (data not shown).

Spleen, lymph nodes, liver, pancreas, kidney, lungs and salivary glands removed from the perfused animals were subjected to macroautoradiography. The autoradiographs of organs from the diabetes-prone and W-

Table 3. Incorporation of ^{111}Indium labelled PBL in various organs of animals previously transferred with Con A-activated splenic lymphocytes

	Percentage of administered dose/g tissue	
	Diabetes-prone BB/W rats[a] ($n=6$)	W-subline rats ($n=6$)
Spleen	8.75 ± 2.35	6.91 ± 1.17
Lung	0.80 ± 0.24	0.78 ± 0.32
Liver	2.44 ± 0.69	1.72 ± 0.35
Pancreas	0.30 ± 0.16	0.22 ± 0.05
Kidney	2.13 ± 0.45	1.39 ± 0.16
Thyroid	0.64 ± 0.29	0.44 ± 0.14
Thymus	0.18 ± 0.05	0.16 ± 0.04

Results expressed as mean ± SD. There were no statistically significant differences between the two groups.
[a] All six animals developed insulitis and two out of six developed diabetes

line animals were visually indistinguishable. Furthermore, the intensity of the images did not differ despite the fact that insulitis was confirmed histologically in the diabetes-prone group.

Incorporation of labelled lymphocytes after passive transfer of activated splenic lymphocytes from diabetic donors

Previous studies have demonstrated that splenic lymphocytes from acutely diabetic rats incubated for 72 h with Con A can transfer diabetes to young diabetes-prone BB/W rats (< 40 days old) but not to W-line rats [17]. The insulitis elicited with this method was studied by the ^{111}indium-labelling technique in two different experiments.

Transfer of concanavalin A stimulated lymphocytes labelled with ^{111}indium. First, Con A-activated lymphocytes were labelled with ^{111}indium and injected into either diabetes-prone ($n=7$) or W-line ($n=4$) animals aged 30–40 days. The animals were sacrified 96 h after injection of 21–28 μCi/10^8 cells. The spleens in both groups had higher activity (32.4 ± 7.9 versus 27.1 ± 8.7%/g, respectively) than the spleens in the experiments using labelled PBL (Table 1), but again there were no differences between diabetes-prone and W-line rats in the incorporation of label into the pancreas. Activity in the pancreas of diabetes-prone rats was 0.56 ± 0.25 versus 1.06 ± 0.77%/g in W-line rats (NS). Histological examination showed no evidence of insulitis or thyroiditis in either group.

Incorporation of successive injections of labelled PBL after the passive transfer of unlabelled Con A-activated cells. In a second protocol, six diabetes-prone and six W-line animals were injected with unlabelled Con A-activated cells immediately after harvesting. PBL were then obtained and labelled twice or three times 2–8 days after the administration of the Con A-activated cells. The average tracer dose was 70–90 μCi/10^8 cells for each injection. The animals were sacrificed 7–10 days after the injection of Con A cells. The activity recovered from various organs is shown in Table 3. There were no differences between the two groups of animals despite the fact that all six diabetes-prone animals developed insulitis and two out of six developed overt diabetes.

Successive scans in vivo of the pancreatic area were also obtained 48 h after the injection of each dose of labelled cells. There were no detectable differences among the pancreas images except for one animal with an area of enhanced imaging suggestive of pancreatic localization. Histological examination of the pancreas from this animal showed global pancreatitis.

Discussion

These studies failed to demonstrate localization of radiolabelled lymphocytes to either the pancreatic islets or the thyroid of BB/W rats. This result contrasts with reports of the utility of labelling peripheral lymphocytes with ^{111}indium in studies of heart transplantation rejection [6] and abscess formation [18]. More importantly, it contrasts with the recent report that labelled peripheral lymphocytes can migrate to and image the pancreas in human Type 1 diabetes [7].

There are many possible reasons for this discrepancy. The islets of Langerhans represent only 1% of the total pancreatic volume. Furthermore, during the spontaneous acute diabetic syndrome in the BB rat, a large number of the islets are end-stage and free of lymphocytic infiltration. It may therefore be difficult to detect sufficient numbers of labelled lymphocytes since the islet population undergoing acute lymphocytic attack is small. But even when insulitis and diabetes were produced using Con A-activated transferred lymphocytes, we were unable to demonstrate labelling of the target islet tissue or recruitment of lymphocytes. Successive scans obtained in animals 48 h following the injection of the labelled lymphocytes failed to demonstrate positive imaging. The only exception was one animal in which pancreatic histological examination at post mortem revealed diffuse pancreatitis in addition to insulitis.

It may be that only a small number of the peripheral lymphocytes are involved in the pathogenesis of diabetes. The selected subpopulation of labelled lymphocytes targeted to the islets may be too small to detect.

The dose of indium used in the majority of our studies was a compromise between the dose of 40 μCi/10^8 cells, above which lymphocyte damage occurs [19] and the minimal one that allows recovery and scintillation imaging. In general, our average percentage recovery per g of organ was similar to that reported from rats 48–72 h after injection [15]. These average values were obtained even in animals that received a labelling dose of 40–100 μCi/10^8 cells, and no correlation was found between the labelling dose and the percentage recovered activity from the organs. The organ which most consistently supports this observation is the spleen.

The only exception in our data was found in animals injected with Con A-activated labelled lympho-

cytes. Damage to the activated lymphocytes, with the release of label and rapid non-specific binding to proteins is the most likely explanation of the high splenic isotopic activity in these animals. It may be that Con A-activated cells are very sensitive to labelling with the ^{111}indium oxine, thus, making them prone to rapid destruction. It has recently been shown that a low labelling dose with ^{111}indium can also damage the DNA of cells [20]. Hence rapid replication of cells in culture with Con A may produce fragile cells susceptible to damage by the label. Irradiation of Con A-activated lymphocytes from acute diabetic animals is known to prevent adoptive transfer [21].

Since the entrapment of labelled lymphocytes within the circulation could mask those within the target organ, we perfused the animals before removal of the organs. Even with this washout procedure there were no differences among groups of animals.

The difference initially observed in the recovery of activity from the thyroid (Table 1) was not confirmed when older animals with a higher incidence of thyroiditis were examined. The reason for this difference is not apparent, but it is possible that in the earlier experiments a population of circulating lymphocytes targeted to the thyroid were labelled. Further experiments are necessary to identify and label a specific subpopulation of lymphocytes targeted to the thyroid. It is of interest to note that positive imaging of the thyroid gland has been obtained in man with autoimmune thyroiditis [22]. The gamma emission of the thyroid was from labelled PBL using a protocol similar to that used in these experiments.

Finally, the positive images of the pancreatic area obtained from patients with diabetes of recent onset [7] were obtained with a technique similar to that used here. In our rat model, no differences in the scan or in direct recovery of activity from pancreas or other organs were observed. Our finding of positive scanning of the pancreatic area in a single animal with diffuse pancreatitis suggests this could be the pathological process observed in the human study. Another explanation may be trapping of the high dose labelled lymphocytes by lymph nodes superimposed on the pancreas.

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Dr. Aldo A. Rossini
Division of Diabetes
University of Massachusetts Medical School
55 Lake North Avenue
Worcester, MA 01605, USA