
Detection of Rejection of Canine Orthotopic Cardiac Allografts with Indium-111 Lymphocytes and Gamma Scintigraphy

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Previous studies have demonstrated the feasibility of detecting canine heterotopic cardiac allograft rejection scintigraphically after administration of ^{111}In lymphocytes. To determine whether the approach is capable of detecting rejection in orthotopic cardiac transplants in which labeled lymphocytes circulating in the blood pool may reduce sensitivity, the present study was performed in which canine orthotopic cardiac transplants were evaluated in vivo. Immunosuppression was maintained with cyclosporine A (10–20 mg/kg/day) and prednisone (1 mg/kg/day) for 2 wk after transplantation. Subsequently, therapy was tapered. Five successful allografts were evaluated scintigraphically every 3 days after administration of 100–350 μCi ^{111}In autologous lymphocytes. Correction for labeled lymphocytes circulating in the blood pool, but not actively sequestered in the allografts was accomplished by administering 3–6 mCi $^{99\text{m}}\text{Tc}$ autologous erythrocytes and employing a previously validated blood-pool activity correction technique. Cardiac infiltration of labeled lymphocytes was quantified as percent indium excess (%IE), scintigraphically detectable ^{111}In in the transplant compared with that in blood, and results were compared with those of concomitantly performed endomyocardial biopsy. Scintigraphic %IE for hearts not undergoing rejection manifest histologically was 0.7 ± 0.4 . Percent IE for rejecting hearts was 6.8 ± 4.0 ($p < 0.05$). Scintigraphy detected each episode of rejection detected by biopsy. Scintigraphic criteria for rejection (%IE > 2 s.d. above normal) were not manifest in any study in which biopsies did not show rejection. Since scintigraphic results with ^{111}In -labeled lymphocytes were concordant with biopsy results in orthotopic cardiac transplants, noninvasive detection of graft rejection in patients should be attainable with the approach developed.

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With improving survival of allograft recipients, cardiac transplantation has become increasingly applicable for treatment of end-stage cardiac disease. Clinical criteria of cardiac allograft rejection, however, are often subtle, especially among patients treated with cyclosporine A (1). As a result, endomyocardial biopsy has become established as the primary means for detecting rejection (2). Unfortunately, repeated endomyocardial biopsy is invasive and expensive. The increasing numbers of recipients requiring sequential evaluation has prompted exploration of noninvasive techniques with

potential power for sensitive and accurate identification of transplant rejection. Results of studies from our laboratory have shown that rejection in heterotopic cardiac allografts can be detected by gamma scintigraphy after i.v. administration of indium-111- (^{111}In) labeled lymphocytes and correction for labeled lymphocytes circulating in the blood pool (3–6). During development of these studies it was recognized that detection of rejection in orthotopic transplants would be much more formidable. Heterotopic allografts perform virtually no external work. They have little blood in the ventricular cavities. In orthotopic transplants, cavitory blood containing circulating labeled lymphocytes could potentially mask uptake in rejecting tissue and mitigate against the utility of the scintigraphic approach. Accordingly, the present study was performed to simulate

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conditions that will be encountered clinically in order to evaluate the feasibility of scintigraphic detection of rejection in orthotopic allografts.

METHODS

Surgical Preparation

Mongrel dogs were subjected to orthotopic cardiac transplantation ($n = 23$). Anesthesia was induced with sodium pentothal (25 mg/kg) and maintained with Enflurane (Ohio Medical Products). Muscle relaxation was facilitated with succinylcholine. Donor hearts obtained from unmatched mongrel dogs were arrested by hyperkalemic crystalloid cardioplegia, excised with the posterior left atrium removed, and placed in iced saline. A right thoracotomy was performed in the recipient and the pericardium opened. Heparin was administered intravenously (100 U/kg bolus, then 50 U/kg/hr while on bypass) after which the left femoral artery was cannulated for cardiopulmonary bypass. Venous return was through the azygous vein and right atrial-inferior vena caval junction. After hypothermia (28°) had been induced, cardiopulmonary bypass was initiated, the aorta was cross-clamped, and the recipient's left and right ventricles were excised.

The donor heart was sutured in place following a conventional sequence of anastomoses. After the aortic anastomosis, rewarming was initiated. The right atrium was opened laterally so that sinus node function would be preserved, and the right atrial anastomosis was performed after which the aorta was unclamped. Defibrillation restored normal sinus rhythm and cardiopulmonary bypass was continued for 25 min to facilitate functional and metabolic recovery of the heart. The animal was weaned from bypass. Anticoagulation was reversed with protamine. The thoracotomy was closed, and hemodynamic and electrocardiographic monitoring was maintained for several hours. Five of the 23 orthotopic transplants performed were successful. The other 18 transplant recipients died perioperatively.

Experimental Protocol

All recipient animals were given methylprednisolone perioperatively (250 mg i.v. bolus) after which cyclosporine A (10–20 mg/kg/day) and prednisone (1 mg/kg/day) were continued for 2 wk to suppress rejection. After 2 wk, immunosuppressive agents were tapered over 5 days so rejection would occur. Autologous lymphocytes were harvested and labeled with ^{111}In beginning 7 days after transplantation. Because of the physical decay of ^{111}In ($t_{1/2} = 2.8$ days) as well as biologic sequestration of labeled cells, blood was harvested for lymphocyte isolation and labeling every 3 days to assure adequate count statistics. Scintigraphy was performed 24 hr after administration of labeled lymphocytes (i.e., every 3 days). Endomyocardial biopsy was performed on the same day as scintigraphy with the use of a Cardiac Biopsy Forceps (Cordis) via internal jugular or femoral vein cutdown. Animals were killed at the onset of clinically evident rejection manifest by frank congestive heart failure or $> 25\%$ diminution in electrocardiographic voltage.

Labeling of Lymphocytes with ^{111}In

Procedures for isolating and labeling lymphocytes have been described previously in detail (5). In brief, 90 ml of

whole blood anticoagulated in acid-citrate-dextrose (ACD) were layered on Ficoll-Hypaque and subjected to density gradient centrifugation for separation of lymphocytes. The lymphocytes were washed with NaCl:ACD (7:1 volume) followed by platelet-poor plasma to remove Ficoll-Hypaque and platelets. The lymphocyte pellet was incubated with 100–400 μCi of [^{111}In]oxine for 20 min. The dose of [^{111}In]oxine approximated 100 $\mu\text{Ci}/10^8$ lymphocytes. Since labeling efficiency was typically 70–85%, in most preparations, administered activity approximated 70–85 $\mu\text{Ci}/10^8$ cells (administered dose generally 100–350 μCi). Cells were then washed with platelet poor plasma to remove unbound ^{111}In . The final lymphocyte preparation contained $2\text{--}4 \times 10^8$ cells of which 84–96% were lymphocytes judging from manual counting on a hemocytometer. The contaminating cells were leukocytes (predominantly polymorphonuclear) and a small number of erythrocytes. The final preparation also contained two to four platelets/lymphocyte. Cell viability, as assessed by the Nigrosin dye exclusion test was 94–97%.

Labeling of Erythrocytes with Technetium-99m

On each day of scintigraphy, 2 ml of whole blood anticoagulated with ACD were obtained. Erythrocytes were separated from plasma by centrifugation and incubated with 50 μg stannous glucoheptonate. The cells were subsequently washed in 0.9% NaCl and incubated with 3–6 mCi of technetium-99m ($^{99\text{m}}\text{Tc}$) pertechnetate for 5 min. They were then washed with plasma to remove unbound $^{99\text{m}}\text{Tc}$. Labeling efficiency was 80–95%. Labeled erythrocytes were administered to the animal after acquisition of ^{111}In scintigrams was complete.

Scintigraphy

Dogs were anesthetized lightly with sodium pentothal and placed in the right lateral decubitus position. Scintigrams were obtained in the straight lateral view with a Siemens gamma camera with a medium-energy, 4,000 parallel hole, large field-of-view collimator set for both peaks of ^{111}In (173 and 247 keV), with a 20% window centered on both photopeaks. Between 300,000–375,000 counts were obtained for each study usually within 30–45 min. Following acquisition of the indium scintigrams, $^{99\text{m}}\text{Tc}$ -labeled autologous erythrocytes were injected i.v. and data from $^{99\text{m}}\text{Tc}$ acquired using a 20% window centered on the 140-keV photopeak. A total of 300,000–375,000 counts were obtained, generally within 3 to 5 min. Radioactivity emanating from liver and spleen was minimized by positioning of the gamma camera. Crossover of radioactivity from the indium to the technetium spectrum was 15%. Because five to ten times as much technetium as indium was administered, the small crossover contamination did not require correction. Data were stored on floppy disks for analysis off-line.

Analysis of Scintigrams

Quantification of ^{111}In lymphocytes infiltrating myocardium was expressed as the percent indium excess (%IE), which reflects the lymphocyte sequestration in the myocardium relative to the activity of indium-labeled lymphocytes in the circulating blood.

For determination of %IE, radioactivity detected from the whole myocardial region of interest (In_{ROI}) was considered to be equal to the radioactivity emanating from labeled lympho-

cytes sequestered in the myocardium (In_{MYO}) plus radioactivity emanating from labeled lymphocytes circulating in the blood pool but not actively sequestered (In_{BP}):

$$In_{ROI} = In_{MYO} + In_{BP} \quad (1)$$

Radioactivity emanating from indium-labeled lymphocytes sequestered in the myocardium (In_{MYO}) is equal to the indium excess (IE). Radioactivity associated with lymphocytes circulating in the blood pool (In_{BP}) is calculated with the use of ^{99m}Tc -labeled erythrocytes which distribute only in the blood pool. A reference region is chosen where the deposition of indium-labeled lymphocytes is unlikely. The ratio of the detected activities for the two radiotracers represents the relative contribution of the two isotopes in the circulating blood pool as detected externally. Thus:

$$In_{BP} = \left(\frac{In_{REF}}{T_{CREF}} \right) T_{CROI}; \quad (2)$$

where REF refers to radioactivity detected from the reference region (in the case of the current study obtained from the region of the ascending or descending aorta or occasionally from the carotid or subclavian arteries). Radioactivity in the myocardium due to sequestered lymphocytes that have left the blood pool (the indium excess, IE) is thus:

$$IE = In_{ROI} - In_{BP} = In_{ROI} - \left[\left(\frac{In_{REF}}{T_{CREF}} \right) \right] T_{CROI}. \quad (3)$$

To normalize IE for differences in administered doses, or differences in attenuation from subject to subject, IE is normalized for In_{BP} thereby expressing the sequestered lymphocytes as a ratio to radioactivity of indium-labeled lymphocytes in the blood (In_{BP}) yielding the percent indium excess (%IE):

$$\%IE = \frac{IE}{In_{BP}} \times 100, \quad (4)$$

where multiplication by 100 gives a percent.

Scintigrams were analyzed off-line with an interactive technique (7). The myocardial region of interest was interactively drawn over the blood pool identified by the labeled erythrocytes thus including myocardium above and below the blood pool but not laterally adjacent to the blood pool. We chose this region for analysis since it was the most objective, conservative one that could be obtained from imaging the myocardium in only one view. Subsequently, the reference region was defined in the same [^{99m}Tc]RBC scintigram corresponding to blood-pool loci exclusively.

Histologic Grading of Biopsies and Direct Determination of Radioactivity in Tissue

All biopsy and tissue specimens obtained at necropsy were evaluated by a cardiac pathologist unaware of scintigraphic results, treatment group, or antemortem physiologic status of the heart. Cardiac samples were graded histologically according to the Billingham criteria (1).

Statistical Analysis

Values were expressed as mean \pm s.d. A t test for independent samples was used for comparison of scintigraphic data in rejecting and nonrejecting hearts. Criteria for significance of differences was $p < 0.05$.

RESULTS

Among the 23 orthotopic canine cardiac transplants performed, five were successful. All nonsurvivors died in the immediate perioperative interval. Survivors were treated with cyclosporine A (10 to 20 mg/kg/day) and prednisone (1 mg/kg/day) for 2 wk after which immunosuppression was tapered. Four recipients were killed when clinical or electrocardiographic criteria of rejection (congestive heart failure or a decrease in QRS voltage) were apparent. Two animals manifested criteria of rejection during the course of immunosuppression.

Time Course of ^{111}In Accumulation

Scintigrams from one dog are shown in Figure 1. The scintigrams obtained during immunosuppression did not manifest criteria of rejection. Endomyocardial biopsy obtained at the same time was negative (no evidence of rejection). Eight days later, following cessation of immunosuppression, scintigrams demonstrated increased myocardial ^{111}In uptake, particularly evident in the subtraction image. Rejection was corroborated by the concomitantly obtained biopsy.

The time course of ^{111}In accumulation for each of the cardiac transplant recipients is shown in Figure 2. In four animals, ^{111}In accumulation increased concomitant with histologic evidence of rejection. One animal died during endomyocardial biopsy while still on immunosuppression therapy at a time when the biopsy was negative for acute rejection.

Comparison of Indium Excess in Hearts Before and at the Time of Rejection

Percent indium excess in scintigrams from hearts before and at the time of rejection is shown in Figure 3. Mean percent indium excess in hearts not manifesting rejection (defined histologically), was 0.7 ± 0.4 (range = 0.1 to 1.3). In contrast, %IE in scintigrams of hearts undergoing rejection was 6.8 ± 4.0 (range = 3.2 to 11.8, $p < 0.05$).

A comparison of endomyocardial biopsy results with scintigraphic data is presented in Table 1. Eight scintigrams were obtained at times when endomyocardial biopsies were negative for rejection. In none was %IE greater than 1.3. Accordingly, all eight did not manifest criteria of rejection [defined as %IE $>$ 2 s.d. above the mean %IE obtained in normal hearts (5)]. Scintigrams of four hearts met the criteria for rejection. Concomitantly obtained biopsies from each reflected mild ($n = 2$) or moderate ($n = 2$) rejection.

DISCUSSION

The results of this study demonstrate that scintigraphy after administration of labeled lymphocytes and correction for blood-pool activity detects rejection in

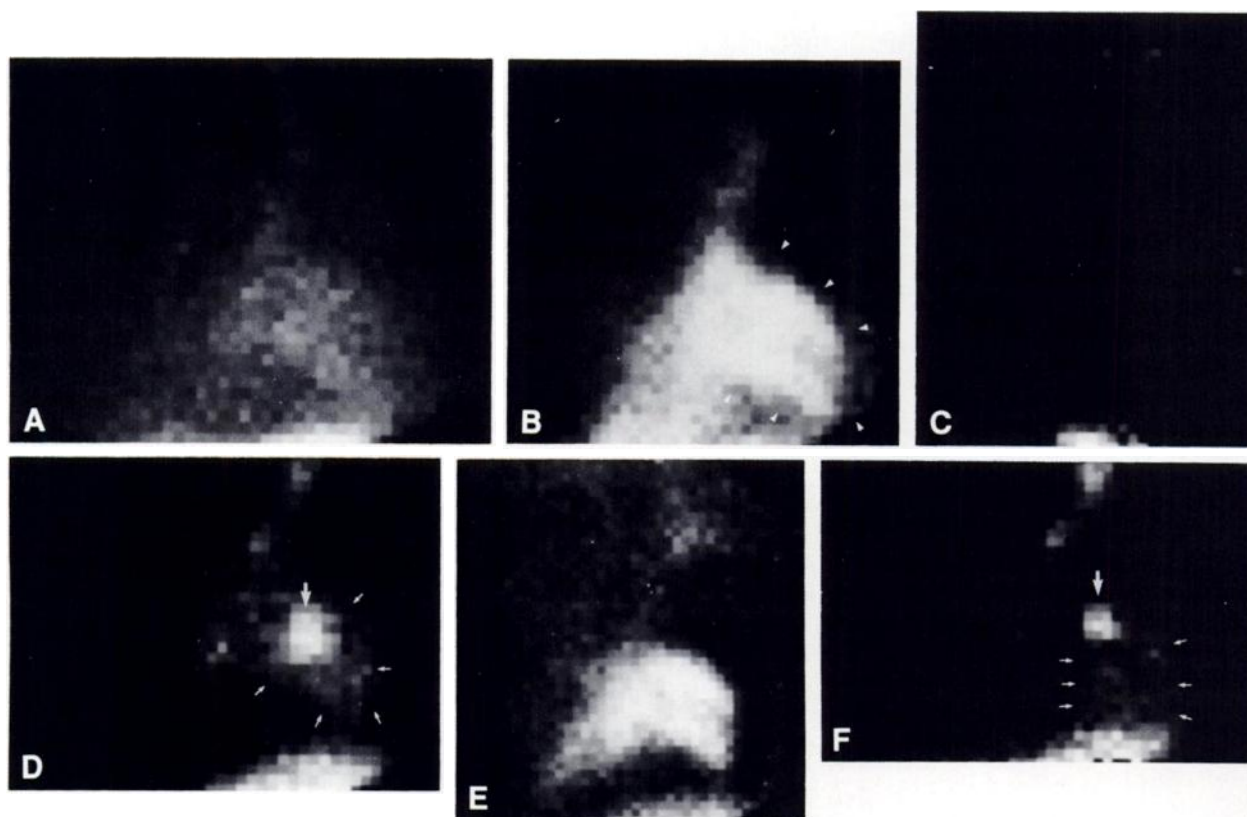


FIGURE 1
Digitized scintigrams from one dog. A–C: Scintigrams obtained 10 days after transplantation (and during immunosuppression) 24 hr after administration of labeled lymphocytes depicting total indium activity (A), [^{99m}Tc]RBCs (B), and the composite scintigram after digital subtraction of lymphocytes circulating in the blood pool (C). The arrows in (B) denote the allograft. No IE was noted at this time. Histology of the endomyocardial biopsy showed no criteria of rejection. D–F: Corresponding scintigrams obtained 8 days later (4 days after immunosuppression had been stopped). Indium excess is evident in the indium scintigram (D) and accentuated in the subtraction image (F) (small arrows). The percent indium excess in the allograft was 7.1. The biopsy at this time was graded as showing mild rejection. Uptake of indium in right atrial thrombus is denoted by the large arrow in (D) and (F).

orthotopic cardiac transplants. Myocardial accumulation of ¹¹¹In was evident in all animals studied at a time when concomitantly obtained biopsy was indicative of rejection, including some still being subjected to immunosuppression. Hearts manifesting scintigraphic criteria of rejection (a %IE > mean ± 2 s.d. of normal, i.e. > 2.2) concordantly manifested histologic criteria of rejection in biopsies. When scintigrams were negative for rejection, concomitant biopsies were devoid of histologic criteria of rejection.

Cardiac transplantation is likely to become increasingly common and increasingly important as a means for treating patients with end-stage cardiac disease. Consequently, noninvasive detection of rejection is particularly desirable. Detection should be sensitive for rejection in its early stages. Even mild episodes of rejection may be associated with diminution of left ventricular function (8). Patients with multiple episodes of rejection are prone to develop irreversible decreases of systolic function (9).

Echocardiography (10,11), signal averaging of QRS

complexes (12), scintigraphy of labeled antibodies directed against myosin (13,14), and magnetic resonance imaging (15) have been advocated for noninvasive assessment of transplant rejection. Echocardiography and electrocardiography suffer from a lack of sensitivity and specificity. Scintigraphy with [¹¹¹In]anti-myosin may be useful, but anti-myosin antibody is taken up primarily or exclusively by necrotic myocardium. Thus, sensitivity for early rejection may be inadequate. Magnetic resonance imaging is promising, but not widely available. The specificity and sensitivity of the technique remain to be determined.

Cells evident earliest by microscopic evaluation in rejecting allografts are mononuclear (16,17). Accordingly, detection of mononuclear cell infiltration offers the potential of detecting rejection in very early stages. Uptake of labeled lymphocytes is a sensitive and specific criterion judging from previous observations with heterotopic allografts (3–6). The purpose of the present study was to determine whether scintigraphy with ¹¹¹In-labeled lymphocytes and blood-pool subtraction could

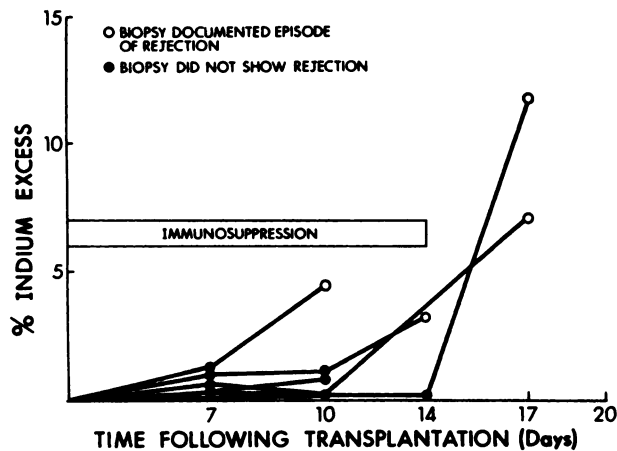


FIGURE 2
Time course of %IE in dogs during the course and after termination of immunosuppression. Time expressed as days after transplantation is shown on the abscissa and %IE on the ordinate. Closed circles indicate scintigrams obtained at times when biopsies did not show rejection. Open circles indicate scintigrams obtained at times when biopsies showed rejection. Myocardial accumulation of ^{111}In was noted in all animals with biopsies indicative of rejection. In two dogs, accumulation occurred during immunosuppression in conjunction with unsuppressed episodes of rejection. One dog died while endomyocardial biopsy was being performed.

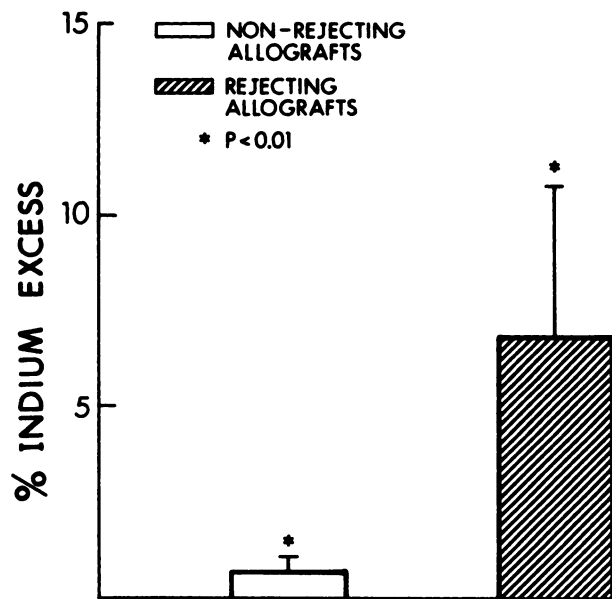


FIGURE 3
Histogram of %IE in scintigrams obtained at times when endomyocardial biopsies showed no criteria of rejection (mean %IE = 0.7 ± 0.4) and at times when rejection was evident histologically (mean %IE = 6.8 ± 4.0 , $p < 0.05$).

detect early rejection in orthotopic cardiac transplant recipients as a prelude to application of the approach in patients. Accordingly, animals were studied under conditions simulating those encountered clinically in

TABLE 1
Comparison of Scintigraphic Results to Endomyocardial Biopsy

Endomyocardial biopsy results	n	Scintigraphic criteria for rejection	No scintigraphic criteria of rejection
Rejection	4	4	0
Normal histology	8	0	8

patients. Complete concordance between scintigraphic and histologic criteria of rejection was observed. Unfortunately, only five of 23 recipients survived the perioperative period. This poor survival, unlike the case for humans, is characteristic of survival in dogs with orthotopic cardiac transplants. Although each surviving animal was studied at multiple time points after the transplant procedure, and as noted above, concordance between biopsy and scintigraphy was striking, the small number of animals studied makes statements regarding the correlation between %IE and histologic biopsy grade difficult. However, in a previous study (6) we demonstrated the correlation between %IE and histologic grade of rejection in 16 dogs with heterotopic thoracic cardiac allografts. The present study was performed to demonstrate that imaging of rejection in orthotopic transplants is feasible when the native heart is not available (in contrast to the case in heterotopic transplants) to serve as a comparison for uptake of labeled lymphocytes. Clearly, ultimate demonstration of the sensitivity and specificity of the technique will require a clinical study in humans.

To correct for lymphocytes circulating in the blood pool but not actively accumulating in the myocardium, we employ a correction scheme that has been previously described for use in both identification of coronary thrombus using ^{111}In platelets (7,18) as well as with our previous work in detecting allograft rejection with ^{111}In lymphocytes (5). Error can be induced by subject motion during the collection of ^{111}In data as well as from motion between collection of data from ^{111}In and $^{99\text{m}}\text{Tc}$. Thus, during scanning, subject motion must be limited, and error is reduced by rapid collection of data from $^{99\text{m}}\text{Tc}$ after collection of ^{111}In data. Although myocardial accumulation of ^{111}In lymphocytes can be observed on nonsubtracted images, they are enhanced using the correction scheme, and when careful attention to data collection is employed, the technique is useful and reliable.

Use of labeled lymphocytes for detection of rejection clinically is not without some disadvantages. Numerous studies have demonstrated that labeling of lymphocytes with [^{111}In]oxine at various concentrations is associated with functional impairment or death of cells, as well as with chromosomal aberrations (19-21). Thakur and McAfee, in a detailed review of the significance of damage induced by radiolabeling (22) calculated that

lymphocytes would receive 350 rad when labeled with 20 $\mu\text{Ci}/10^8$ cells. When 10^8 leukocytes, accompanied by $20\text{--}30 \times 10^6$ lymphocytes are labeled with 500 μCi of [^{111}In]oxine (the dose currently suggested as a maximum for most labeling applications), lymphocytes receive $\sim 8,750$ rad, a radiation burden clearly associated with functional and structural damage and with cell death. The concern of some authors are that chromosomal abnormalities may result in oncogenic potential (19). As noted by Thakur and McAfee (22), spontaneous chromosomal aberrations increase linearly with age, and are also associated with a number of therapeutic and diagnostic procedures including chemotherapy, radiation therapy, and diagnostic radiology. Nonetheless, Thakur and McAfee concluded that when 10^8 lymphocytes (0.01% of the body's total lymphocyte pool) are labeled with 100 μCi of ^{111}In , the excess risk of a fatal malignancy over 30 yr would approximate 1 per million. Clinical experience with ^{111}In -labeled lymphocytes is limited, and close follow-up is warranted. Use of lymphocytes labeled with shorter-lived radionuclides or labeling of cells with surface markers (i.e., anti-lymphocyte antibodies) may obviate some of the potential limitations.

Uptake of labeled lymphocytes into myocardium detected scintigraphically cannot be construed to be an entirely specific phenomenon for detection of rejection. Diseases associated with lymphocytic infiltration of the myocardium such as toxoplasmosis and cytomegalovirus myocarditis (both of which are rare but significant complications of immunosuppression) are likely to be detected as well (23,24). However, these myocarditides are usually associated with systemic disease and serologic abnormalities. If they are suspected, endomyocardial biopsy may be required for differential diagnosis. Unfortunately, characteristic changes of toxoplasmosis (cysts) or cytomegalovirus (inclusions in megalic cells) may not be apparent.

Recent preliminary results from our laboratory suggest that the scintigraphic technique developed permits detection of rejection at an early enough time that the process can be reversed by salvage immunotherapy (25). Thus, scintigraphy with labeled lymphocytes appears likely to be capable of detecting rejection when therapeutic interventions can still be effective. Accumulation of ^{111}In -labeled lymphocytes occurs progressively with time during the course of rejection. Thus, the technique developed may be particularly useful in determining appropriate timing of endomyocardial biopsy and in clarifying the significance of histologically equivocal biopsy results in patients with cardiac allografts.

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