QUANTITATION OF TRANSPLANTATION IMMUNITY*, ‡

I. METHOD

BY JOHN S. NAJARIAN, M.D., AND JOSEPH D. FELDMAN, M.D.

(From the Experimental Surgery Laboratories, University of California Medical Center, San Francisco, and the Division of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California)

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Investigation of the basic mechanisms of transplantation rejection has been hampered in the past by the imprecise techniques available to determine transplantation immunity. These techniques have included the gross and microscopic estimation of survival of skin and organ grafts (1) and, more recently, the "delayed" intradermal skin test described by Brent *et al.* (2). Results obtained with these methods have been interpreted subjectively and their evaluation may be considered to be qualitative rather than quantitative.

Several procedures to quantitate transplantation immunity recently have been proposed. All of these methods depend on the measurement of antibody formation by cells passively transferred either to homologous recipients (3), or to tolerant recipients along with "killer" cells (4), or by plating antibody-forming "target" cells and killer lymphoid cells on agar (5), as originally described by Jerne and Nordin (6).

The purpose of this investigation was to determine whether direct quantitation of transplantation immunity was possible by measuring the disappearance rate of lymphoid cells (antigen) labeled with H^3-thymidine.

Materials and Methods

Mice of the A, CBA, and BALB/C strains were used throughout these experiments.

Preparation of Labeled Cell Suspensions.—Lymphoid cells were labeled *in vivo* by injecting donor mice with 0.5 μc of tritiated thymidine per gram body weight every 12 hours for a total of 7 injections. Six hours after the last isotope injection, donor spleens were removed and raked in balanced salt solution (Hanks'). The resulting suspension was passed through 100 mesh nylon gauze, and after counting, was adjusted to 2×10^8 cells per ml. A 0.2 ml aliquot was removed for determination of total radioactivity (7) and another aliquot was used to prepare autoradiographic smears to record the percentage of labeled cells.

Cellular Distribution.—A standard dose of 1×10^8-labeled lymphoid cells in 0.5 ml volume

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was injected intravenously into isologous and homologous mice. Three animals were sacrificed at each appropriate time interval from 10 minutes up to 100 days. Total radioactivity was measured in the spleen, lymph nodes, thymus, and bone marrow, in aliquots of all thoracic and abdominal viscera, of blood, urine, and feces, and of carcass tissues such as skin and muscle. The method of organ preparation and isotope counting has been described previously (7).

The results of these distribution experiments in both isologous and homologous recipients, revealed that the total radioactivity and number of labeled cells were maximal in the periph-

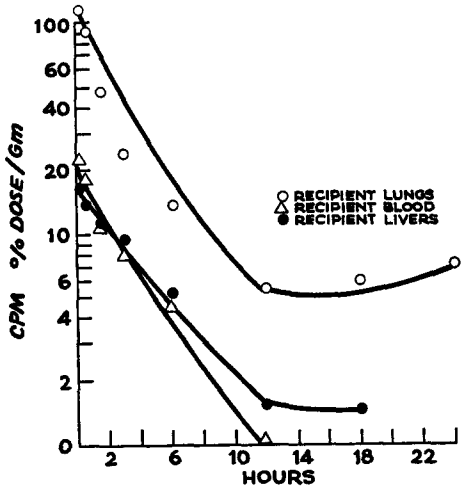

FIG. 1. Semilogarithmic plot of the disappearance rate during the first 24 hours of radioactivity in the blood, liver, and lungs of isologous or homologous mice injected intravenously with H^3-thymidine-labeled cells. There is a rapid decline to less than 0.1 per cent (blood), 1 per cent (liver), and 6 per cent (lungs) of the dose per gram of tissue, at 12 hours. Beyond this time radioactivity remains fairly constant and low in these organs throughout the period of observation.

eral blood immediately after transfusion and decreased in a straight-line exponential fall over the next 12 hours (Fig. 1). Thereafter, only an occasional labeled cell could be found by autoradiography in peripheral blood. Similar findings were obtained from other non-lymphoid parenchymal organs (Fig. 1) and might have represented, to some extent, the blood within those organs. Recovery of radioactivity from the bone marrow was erratic and might have been due to the dynamic influx of labeled cells into and out of the bone marrow, to the difficulty of weighing accurately such small amounts of tissue, or both (Fig. 2).

After infusion of H^3-labeled cells, total radioactivity in the spleen and lymph nodes rose to a maximum within 12 and 24 hours, respectively, and disappeared exponentially in isologous recipients over a period of 10 to 14 days and in homologous recipients over a period of 5 to 7 days (Figs. 3 to 6). Since the spleen was removed more easily, and lacked adventitial tissue, this organ was selected for measuring loss of transplanted labeled cells. The disappearance of radioactivity in lymph nodes was determined only to confirm the results obtained from spleen counts.

Detailed analysis of organ distribution of passively transferred labeled cells in isologous and homologous mice with this technique will be reported in a subsequent publication.

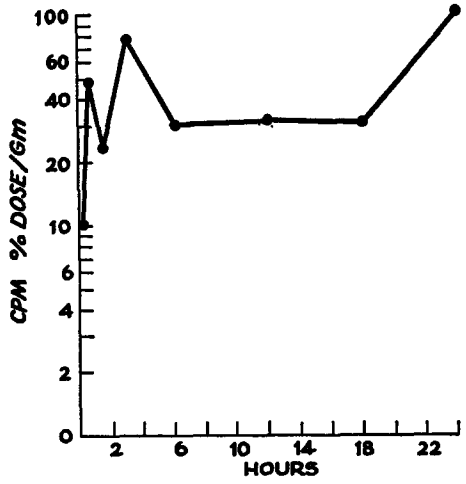

FIG. 2. Semilogarithmic plot of radioactivity in bone marrow of isologous or homologous mice injected intravenously with H^3-thymidine–labeled cells. There are wide variations in concentration of isotope in this organ during the first 24 hours.

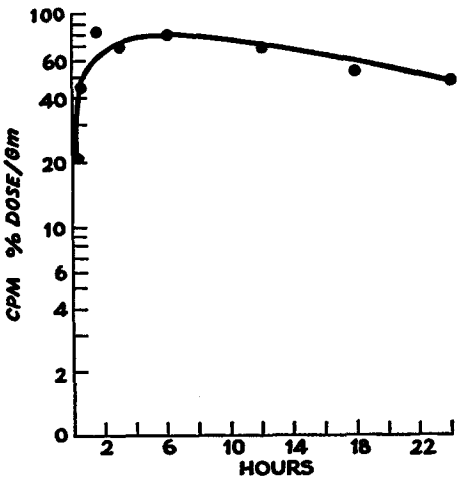

FIG. 3. Semilogarithmic plot of radioactivity in the spleens of isologous or homologous mice injected intravenously with H^3-thymidine–labeled cells. A peak is reached within 6 hours, followed by an exponential decline after 12 hours (see also Fig. 8).

Autoradiographs of cell suspensions, blood, and histologic sections of recipient organs were prepared with NTB2 liquid emulsion technique and hematoxylin staining.

Isologous Experiments.—Labeled A spleen cells were injected into A recipient mice, and total radioactivity was determined in the spleen and lymph nodes of groups of 3 mice for each interval of time. In 11 trials, A mice were used, primarily because of the ease of intravenous

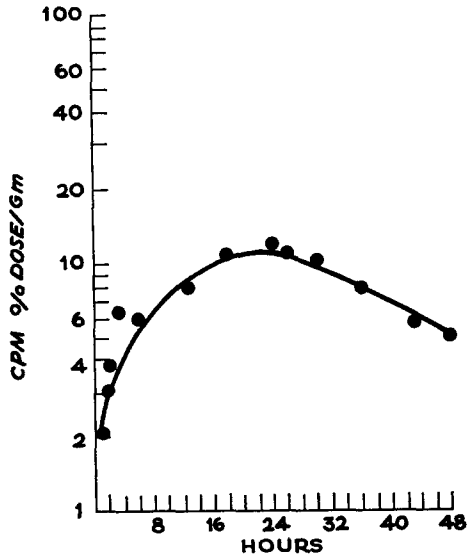

FIG. 4. Semilogarithmic plot of radioactivity in the lymph nodes of isologous or homologous mice injected intravenously with H^3-thymidine–labeled cells. A peak is reached within 24 hours, followed by an exponential decline.

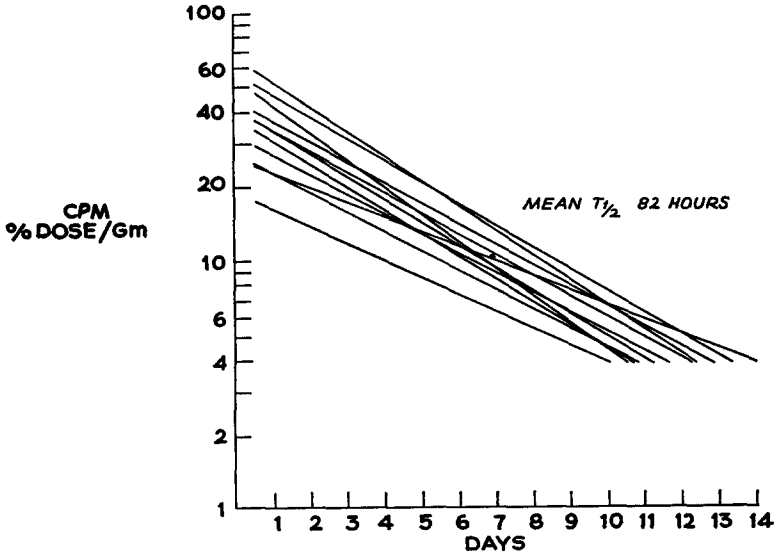

FIG. 5. Semilogarithmic plot of disappearance rates of radioactivity in the spleens of *isologous* mice injected intravenously with H^3-thymidine–labeled cells. There is an exponential decline from 12 hours to between 10 and 14 days. Mean half-life of isotope, 82 hours.

injection; in 5 trials, labeled CBA cells were injected into CBA mice to determine the rate of decay of radioactivity in a second isologous system.

Homologous Experiments.—Labeled CBA cells were injected into A mice in 12 of the experiments; the reciprocal experiment with A-labeled cells infused into CBA mice was also performed in 6 runs. To determine the effect of transplantation immunity across another H-2 barrier, BALB/C-labeled cells were injected into both A (3 trials) and CBA (3 trials) mice and the recovery of radioactivity from the recipient spleens was determined.

Homologous Sensitized Mice.—Recipient mice (predominantly A strain) were immunized by CBA skin homografts and by intraperitoneal injection of 2×10^7 homologous CBA spleen

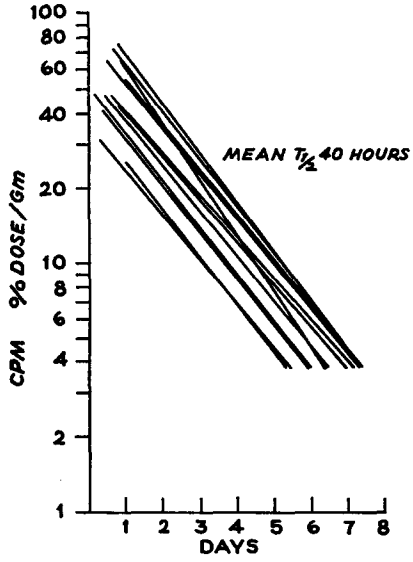

FIG. 6. Semilogarithmic plot of disappearance rates of radioactivity in the spleens of *homologous* mice injected intravenously with H^3-thymidine–labeled cells. There is an exponential decline from 12 hours to between 6.0 and 7.5 days. Mean half-life of isotope, 40 hours.

cells 2 days later. Ten days after grafting, the mice were injected with labeled CBA cells (6 trials). In other experiments, labeled A cells were administered to CBA mice immunized with A tissues (4 trials) and labeled BALB/C cells to A mice immunized with BALB/C tissues (2 trials).

Calculation of Results.—After the samples were counted in a liquid scintillation counter, an internal standard (25 μl of tritiated thymidine containing approximately 25,000 CPM) was added to each vial to determine the degree of quench. The formula used was:

$$\frac{S - (C_2 - C_1)}{S} \times 100$$

where S was internal standard, C_2 was the count of the sample after addition of the standard, and C_1 was the count of the sample before addition of the standard. Background of the counter ranged from 15 to 20 CPM at a voltage of 1160 with efficiency of 17 per cent. After correction for background and quenching, results were recorded as counts per minute per gram of tissue

expressed as a percentage of dose. In addition, in order to standardize the calculations, the body weights were corrected to yield a standard weight of a 25 gm mouse. Thus, the final figures were expressed as a percentage of the dose of injected radioactivity per gram of tissue in a 25 gm mouse. This permitted a comparison of all samples of all experiments. A minimum of 3 animals was used for the calculation of each point, and the individual points were plotted on semilogarithmic paper. From the graph of the regression slopes, the half-life ($T\frac{1}{2}$) of the radioactivity was expressed in hours.

Calculation of the regression slope (b) was made with the formula:

$$b = \frac{xy - \frac{xy}{N}}{x^2 - \frac{(x)^2}{N}}$$

where x was days, y was the log of percentage of dose and N was the number of observations. The P values of differences between individual log slopes were obtained to determine statistical significance.

RESULTS

Isologous Experiments.—Radioactivity from isologous spleens reached a maximum 6 hours after transfusion, remained at this level for another 6 hours, and then declined in a linear exponential fashion (Fig. 5). The phase of exponential decline thus began some 12 hours after cell transfer, when radioactivity in the spleen (average weight, 100 mg) was between 4 and 6 per cent of the dose, and continued for 10 to 14 days, when radioactivity in the spleen fell to between 0.3 to 0.4 per cent of the dose and remained at this level for up to 100 days (Fig. 8). As noted in Fig. 5, the half-lives of isotope disappearance ranged from 60 to 92 hours, with a mean of 82 hours. The variation was attributed to the use of different pools of labeled cells in each experiment.

Homologous Experiments.—The data from 12 CBA spleen cell transfers into A recipient mice are shown in Fig. 6. The maximum radioactivity, ranging from 2.5 to 8 per cent of the dose, was recovered at 12 hours; it regressed linearly to the 6th and 7th day. The exponential phase terminated usually at about 0.4 per cent of the injected dose per spleen and thereafter remained fairly constant at this level up to 50 days (Fig. 8). The half-lives of the exponential decay of radioactivity ranged between 36 to 44 hours with a mean $T\frac{1}{2}$ of 40 hours. This result was obtained when different donor pools were used in different experiments.

The mean $T\frac{1}{2}$ of radioactivity in spleens of CBA mice following injection of labeled A cells was 39 hours. The $T\frac{1}{2}$ obtained across two other H-2 histocompatibility differences using labeled BALB/C cells injected into A and CBA recipients was 37 and 39 hours, respectively. Thus, a remarkably similar disappearance rate of transferred homologous cells between H-2k, a and d differences was observed, and the variations from the mean of 40 hours were small and within the 99 per cent confidence limit.

Three homologous experiments, each done in triplicate, from the same pool of labeled cells, showed that the half-disappearance rate of the transferred cells did not vary more than 1 hour. Therefore, half-life determinations of isotope from the same pool of labeled cells could be considered significant if the difference was greater than 3 hours and highly significant if the difference was greater than 4 hours.

Homologous Sensitized Mice.—The data from four experiments are shown in Fig. 7. Recovery of radioactivity was recorded from spleens of A mice (sensi-

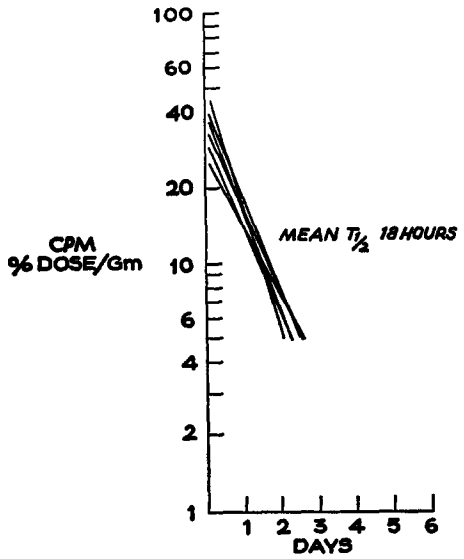

FIG. 7. Semilogarithmic plot of disappearance rates of radioactivity in the spleens of *homologous sensitized* mice injected intravenously with H^3-thymidine–labeled cells. There is an exponential decline from 4 hours to between 2.5 to 3 days. Mean half-life of isotope, 18 hours.

tized to CBA mice) into which labeled CBA spleen cells were transferred. Radioactivity reached a maximum of 3 to 4 per cent of the dose per spleen within 3 to 4 hours after transfusion of labeled cells and disappeared exponentially over a 2 to 3 day period to a level of about 0.5 per cent of the dose per spleen. The half-life of this first exponential phase was 18 hours. Fig. 8 illustrates that the loss of labeled cells in presensitized hosts was a complex process, and following the first phase there was a second 2 to 3 day period of exponential loss with a half-life of 80 hours. Five days after infusion, the line of regression almost paralleled the baseline, and like the curves in the other two situations, remained fairly constant for the duration of observation.

The mean half-life of the radioactivity of the first linear decline in the spleens

of sensitized CBA mice injected with labeled A cells and of sensitized A mice injected with labeled BALB/C cells was 20 and 16 hours, respectively.

The linear disappearance rate of transfused labeled cells in the isologous, homologous, or presensitized homologous experiments was significantly different from the other two, with P values of less than 0.01 (Fig. 8).

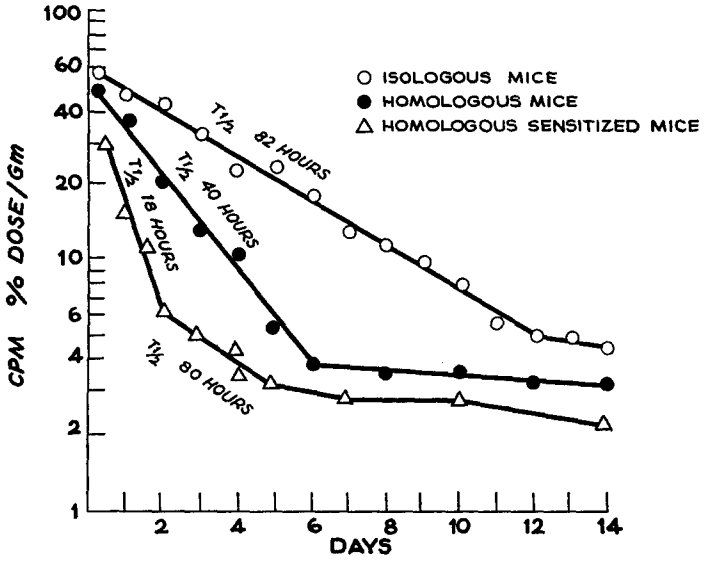

FIG. 8. Comparison of the disappearance curves of radioactivity in the spleens of isologous, homologous, and homologous sensitized mice injected intravenously with H^3-thymindine–labeled cells. Following the linear loss of isotope, radioactivity in the spleens remains at a fairly constant low level, up to 100 days. In the homologous sensitized group, there is an initial exponential decline with a half-life of 18 hours, a second exponential decline with a half-life of 80 hours, and after 5 days the isotope level parallels the baseline at low concentration.

DISCUSSION

The method described in this paper was originally developed to see whether the interaction of labeled cells, as antigen, and homologous recipient might be comparable with the technique of observing antigen elimination using isotopically labeled serum proteins (8). In the latter system, antigen first equilibrates rapidly between intra- and extravascular spaces of the host and then slowly disappears until antibody formation causes rapid removal of the antigen from the injected recipient. It was apparent that transfused foreign labeled cells were treated in a different fashion. The tagged cells were quickly lost from the circulating blood and, indeed, from the whole body, and less than 20 per cent remained at 24 hours (9). However, in the spleen and lymph nodes, radioactivity

accumulated to a maximum within the first 12 to 24 hours. Thereafter, the isotope was eliminated exponentially to a level of about 0.5 per cent of the dose per spleen, at which level radioactivity remained for prolonged periods of time. There was no sharp increased loss of isotope to indicate the time that immune elimination occurred. Instead, the slope of isotope disappearance from the spleen expressed the state of primary or secondary immunization.

The exponential loss of labeled cells in a homologous recipient (equivalent to a first-set skin homograft rejection) was completed in 6 to 7 days; the loss in a presensitized recipient (equivalent to a second-set skin homograft rejection) was completed in 2+ days. These times were less than those reported for first-set (10 to 11 days) and second-set (6 days) skin homograft destruction. One might question whether the isotope technique of measuring antigen (cells) elimination from the spleen was recording the same immunologic phenomena that occurred in solid graft destruction. This question could not be answered directly. However, the differences in rejection times might be reasonably explained. The intravenous administration of labeled cells into a recipient permitted antigen (cells) to reach the recognition centers of the host almost immediately. Further, once the immune process was activated, the antigen was on location, to be disposed of promptly. In contradistinction, the process of destruction of a first-set skin homograft requires some 24 hours for the development of vascular connections with the host and additional time for antigen to reach the recognition centers in effective amounts. Once the immune process has been established, the effective agents of rejection must be delivered to the graft. Indeed, the beginnings of skin graft destruction have been visualized microscopically 6 to 7 days after grafting, and completion of the process is achieved 4 to 5 days later. On the other hand, rejection of whole organs grafted by vascular anastomosis is accomplished in 6 to 7 days, probably because foreign transplantation antigens enter the circulating blood when anastomosis is completed. In previously sensitized hosts, whole organ grafts, particularly kidneys, are rejected 1 to 2 days after transplantation, a time period corresponding to that observed when labeled cells were transfused into preimmunized recipients.

The system of transfused labeled cells offered the advantages of precision and sensitivity, as compared with the estimation of skin homograft survival. When a single pool of labeled cells was used in homologous or presensitized recipients, the slopes of the disappearance curves of spleen radioactivity were fairly constant and half-lives did not vary by more than 1 to 2 hours. The difficulty of estimating survival times of skin homografts has been frequently noted. The evaluation of gross or microscopic alterations of the graft, either early or later in the process of rejection, has been a relatively poor end-point to measure and has necessitated a good deal of experience in addition to subjective criteria. Furthermore, the effect of weak antigens, *e.g.* H—3 differences, or Y antigen, or of altered host responses, *e.g.* by drugs or irradiation, have

been clearly recorded by slight shifts in the disappearance curves of labeled cells (9), while the end-point of graft destruction under similar circumstances has been nebulous. An illustration of the sensitivity of the isotope method was shown by the decrease of T½ of 40 hours in recipients of homologous labeled cells to T½ of 35 hours in recipients of homologous labeled cells from the same pool and of isologous serum from mice immunized against these cells. Solid skin homografts were not affected by the infusion of immune serum (10).

For optimal and reproducible results the technique may be summarized as follows. A single pool of labeled homologous cells is used to infuse both control and experimental groups of 9 mice each. Each point on the curve of linear disappearance of radioactivity is calculated from the isotope content of the spleens of 3 recipient mice. In the homologous experiments, only 3 points are needed between 12 hours and 6 days after infusion. In the presensitized homologous situation, 3 points are plotted between 6 and 48 hours. A difference of half-lives between control and experimental groups of more than 3 hours is significant.

The transfer of labeled cells as described here into isologous and homologous hosts has raised several questions about some of the observations. In the spleens of isologous recipients, the mean half-life was 82 hours, or stated differently, more than 90 per cent of the transferred cells was destroyed or lost in 23 to 24 days (from 4 to 0.4 per cent); about 0.5 per cent of the radioactivity administered could be found in the spleen up to 100 days after injection. Did this represent two populations of lymphoid cells, the majority surviving for several weeks and a small minority having a long life? Did the persistence of isotope represent reutilization of the H^3-thymidine, or did it represent a low level of flow into the spleen of cells with isotope, from other sources? Was the half-life of 82 hours the result of increased population pressure in the lymphoid organs as a result of injecting 1×10^8 cells into a mouse, or would the half-life be prolonged if there were more "lebensraum"? In homologous recipients, the slope of disappearance of isotope at 24 hours was significantly steeper than that in isologous hosts. Did this represent an early 24 hour immune response, comparable to that observed when phage antigen has been used (11), or was this unrelated to immunologic phenomena? In presensitized homologous recipients, there were two linear phases of isotope disappearance. The first occurred 6 to 48 hours after transfusion (with a half-life of 18 hours) and most likely represented destruction of the labeled cells. The second, occurring between 2 and 5 days, with a half-life of 80 hours, was not readily explained. It might have been due to overloading of the reticuloendothelial system with delayed disposal of cellular elements, or it might have represented an accelerated rate of H^3 reutilization which diminished to a constant low level after 5 days.

SUMMARY

A technique for *in vivo* quantitation of transplantation immunity has been presented. Known numbers of H^3-thymidine–labeled lymphoid cells were

injected intravenously into isologous or homologous recipients. The total radioactivity in host spleens was counted at different time intervals. The rate of isotope disappearance from the spleen was exponential to 0.5 per cent of the dose or less. In isologous recipients, the calculated half-life of labeled cells was 82 hours; in homologous recipients it was 40 hours; and in presensitized homologous hosts it was 18 hours. The technique is a highly reproducible, precise, and sensitive measure of transplantation immunity.

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