The Separation and $^{51}$Chromium Labeling of Human Lymphocytes With In Vivo Studies of Survival and Migration

By Peter Hersey

This study looks at the application of $^{51}$Cr labeling of lymphocytes as a method of obtaining in vivo information about the lymphocyte in human beings. Lymphocytes were separated from whole blood by methods based on isopycnic and rate zonal centrifugation techniques and the conditions for $^{51}$Cr uptake by the separated lymphocytes standardized to enable a known amount of radioactivity to be injected into the subjects under study. The uptake of the label into various sites in the body was studied by the means of surface probes linked synchronously to a digital printout device and the survival in the circulation estimated by scintillation counting of blood samples taken at various times after injection of the label. The in vivo studies of survival and migration in 10 normal subjects show an initial rapid clearance of cells from the circulation associated with an uptake of cells into spleen and liver sites, and to a lesser extent, into sites over bone marrow and the abdomen. Survival of the circulating lymphocytes after this period appears to be relatively short, with a half-life of 1.7 days. As the available evidence suggests, this short life may be due to the differential trapping of short-lived lymphocytes in the circulation at the expense of the long-lived lymphocytes. Kinetic interpretations of the data indicate an inverse exponential uptake of cells into the sites studied, and the decline over the organs appears to follow the death rate of the cells in the body as a whole. Comparisons with studies in patients having chronic lymphatic leukemia show a relative inability of leukemic lymphocytes to leave the circulation and enter some sites in the body. These preliminary studies indicate the potential of $^{51}$Cr labeling as a useful clinical research tool in the study of lymphocytes in human beings.

Although a number of different methods for the study of lymphocytes have been introduced in recent years, very few of these are able to provide in vivo information about lymphocytes in human beings. In particular, there are virtually no methods at present available whereby kinetic data about lymphocytes in humans can be obtained equivalent to the studies of Gowans and co-workers in the rat or of Bainbridge et al. in mice. Perry et al. have studied the traffic of tritium-labeled lymphocytes in the thoracic duct of patients cannulated at operation, but obviously these techniques are not generally applicable for clinical studies.

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Over the past two decades $^{51}$Cr labeling of red cells and platelets has provided suitable methods for in vivo studies of these two cell types, but with the exception of some earlier studies on leukemic lymphocytes these techniques have not been applied to the study of lymphocytes because of the difficulty in obtaining sufficient numbers of lymphocytes in a pure suspension from the blood of human subjects. More recently, a number of studies on $^{51}$Cr labeling of mixtures of leukocytes have been reported but, with the exception of those carried out by Pfisterer et al., the resultant data are difficult to interpret because of the various cell populations labeled. In the current study a pure preparation of lymphocytes in terms of $^{51}$Cr uptake has been obtained, and thus the difficulty in interpretation inherent in these studies is avoided.

In the conventional $^{51}$Cr studies on red cells and platelets, attention has generally been paid to the survival and sequestration of these cells in various regions of the body. Similar studies have been made in this work, but in addition an attempt to obtain information about the kinetics of these processes has been made, as it is clear from experimental work in animals that the movement of these cells within the body is important in the overall immunological function of the body.¹

**Materials and Methods**

**Subjects**

Ten normal male subjects were studied with ages ranging from 23 to 41. These subjects were members of the hospital staff and final-year medical students during their clinical studies. Three male patients (ages 68 to 73) and one female patient (age 70) with chronic lymphatic leukemia (CLL) were also studied and are included in this report for comparative purposes. Two subjects were untreated and two were being treated with prednisolone and chlorambucil. Informed written consent was obtained from all patients and informed consent from the normal subjects.

**Separation of Lymphocytes**

The method of separation of lymphocytes from whole blood has been adapted from that described by Perper, Zee, and Michelson based on isopyknic and rate zonal methods of centrifugation. The theory underlying these methods is described by Boone et al. In normal subjects approximately 450 cc of venous blood is collected from the donor into a sterile tripack container (Tutu Laboratories, Lane Cove, New South Wales) containing 5000 units of heparin and 80 ml of plasmagel (Lab. Roger Bellon, Neuilly, France). In patients with CLL only 100-200 ml of whole blood was necessary to supply sufficient lymphocytes for the test. Sedimentation is allowed to occur for 3 hr, and the supernatant expressed into the second bag of the tripack container. Centrifugation of the leukocyte-rich plasma is carried out at 350/g for 10 min and the supernatant once more expressed into the third bag of the tripack container. (This plasma is later centrifuged to remove the platelets and is used for the subsequent washing procedures.) The cell button is resuspended in a 50% mixture of saline and plasma within a sterile syringe and laid carefully onto the surface of 10 ml of hypaque solution (Wintnrop, Sydney) having a specific gravity of 1.078 in a sealed sterile McCartney bottle. This is centrifuged at 400 g at the interface for 20 min, and the lymphocytes on the top of the hypaque solution are removed with a sterile syringe and carefully laid over 17 ml of a hypaque solution having a specific gravity of 1.036 in a sterile McCartney bottle. Centrifugation at 200 g at the bottom of the tube is carried out for 15 min and the lymphocytes at the bottom of the tube from this step are then resuspended in approximately 10 ml of saline for the labeling procedure. Sterility is
Fig. 1.—The optimum conditions for 51Cr uptake by human lymphocytes, determined at 37°C, with an incubation period of 1 hr unless shown otherwise.
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maintained by use of a closed system throughout the separation procedure and by the use of sterile plastic disposable material where possible.

51Cr Labeling

The various factors found to be of importance in the labeling procedure were determined by carrying out a series of experiments on the lymphocytes from five normal subjects in which only one factor at a time was varied and all other factors were kept constant. Figure 1 illustrates the results of these preliminary experiments, and all subsequent labeling procedures adhered to the optimum conditions as determined by these studies. The volume of the incubation suspension was adjusted to give cell concentrations in the region of 20-50 million cells/ml and was usually approximately 8 ml. 51Cr was added to give a final concentration of 50 to 100/MCi ml and in no cases did the Na251CrO4 concentration exceed 2.5 μg/ml. Incubation was carried out at 37°C for 1 hr and the incubated cells were washed twice in plasma before being resuspended in 20 ml of plasma for return to the subject. The time taken from venesection to reinjection of the cells was approximately 3% to 4 hr.

Cell Viability

Cell viability was assessed in vitro by the trypan blue dye exclusion test after the method used by Tennant11 and Black et al.12 in which the test lymphocytes in a solution of 10% plasma in saline were mixed in an equal volume of 0.2% trypan blue in saline. The cell suspension was incubated for 8 hr at 37°C and the number of cells taking up the dye were counted at a magnification of 400 times using a hemocytometer chamber. In all studies carried out the viability assessed by this method exceeded 95% prior to the reinjection into the subjects. In addition to the in vitro viability assessment, in vivo viability was assessed by estimating the 51Cr excretion in 24-hr collections of urine. These were carried out for the first 8 days of the study and the samples were counted in a large volume, lead shielded, Gamma Counter with 3-in. iodide crystal above and below the sample (Ekco 5000-type series). Similar volume and spatial characteristics were observed in the counting of all specimens.

Table 1 illustrates the results of these studies for the normal and CLL subjects. Also included are the 24-hr urinary excretion rates after the injection of heat-killed, washed cells into three normal subjects and the mean excretion rates from two subjects injected with frozen and thawed cells including the supernatant activity released by the freezing-thawing process. Fecal collections for 1 wk in two subjects did not show any significant excretion of radioactivity in the fecal samples and was discontinued in subsequent studies.

In Vivo Studies

The suspended lymphocytes were returned to the circulation over a 15-20-sec period and blood samples taken from the other arm, via an indwelling catheter (Dwellcath., Tutu Laboratories) and a three-way plastic disposable tap (Pharmaseal, Glendale, Calif.) at 2,
5, 15, and 30 min and at 1, 2, 3, and 4 hr during the initial period of the study and on each subsequent day for the duration of the study.

Cells from the 10-ml blood samples were washed twice in saline and counted in a conventional well-type sodium iodide crystal scintillation counter (Nuclear-Chicago, Model 1087).

Several of the cell samples at varying time intervals from each study were further separated by the methods described and in each case activity was detected only in the lymphocytes.

Body surface counts were determined over the liver, spleen, iliac crest, central abdomen, and upper right lung field by the use of four separate probes (Phillips P. W. 4119) containing shielded 2-in. thallium activated sodium iodide crystals with “flat field” collimators (Phillips XL6000) the 50% isosensitivity lines of which encompass a field 5 cm deep and 8 cm wide in air. The probes were linked to a synchronous printout system which printed out the count rate over 100 sec for each organ so providing a continuous synchronous digital record of the organs under study.

Surface counts were begun immediately after injection of the labeled lymphocytes and continued for 4 hr or until the maximum activity had been reached over the spleen. Counting was continuous for the first hour of the study and then for 15-min periods hourly thereafter. Urine was voided after the first hour of study to avoid interference with the activity recorded by the probe over the iliac crest from radioactivity voided in the urine. Daily counts were recorded over the first 8 days and on alternate days thereafter until the end of the second week. All surface counts are expressed relative to 1% of the injected radioactivity measured in a constant volume at the surface of the probes.

RESULTS

Separation of Lymphocytes From Whole Blood

Table 2 illustrates the yield of lymphocytes from whole blood where close to 50% of the available lymphocytes are obtained. This table further illustrates that most of the lymphocytes are lost in the sedimentation of red cells and that close to 70% recovery is obtained from the rate zonal and isopyknic centrifugation steps of the procedure. This yield is less than that of 80% obtained by Perper et al.9 using similar methods on smaller volumes of blood but compares favorably with the 52% recovery obtained by Noble and Cutts13 using Ficoll gradients and small volumes of blood.

Other methods using columns and hypotonic lysis of red cells have in most hands6,14 given yields in the vicinity of 20–30%, and contamination with red cells have made these methods unsuitable for 51Cr labeling studies. The method reported by Woods14 in which a 60% yield is claimed has also been found unsuitable for 51Cr labeling studies because of the contamination with red cells and polymorphs.

The purity of the final lymphocyte suspension was assessed from stained smears and by assessing the size profile of the final suspension with the

<table>
<thead>
<tr>
<th>Lymphocytes in 450 cc of Whole Blood (x 10^9)</th>
<th>Yield after Sedimentation of RBC</th>
<th>Final Yield x 10^9</th>
<th>Percentage Yield from Sedimented Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1015</td>
<td>682</td>
<td>436</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>234</td>
<td>217</td>
<td>125</td>
</tr>
</tbody>
</table>

* Data from 10 normal subjects.
Coulter Counter. The only contaminant that has been found in the final preparations apart from occasional monocytes are platelets, which have been found to occur in numbers up to, but usually less than, the equivalent number of lymphocytes. Since the equivalent uptake of $^{51}$Cr by platelets is only 1% that of lymphocytes, this contamination is not regarded as significant in the current study.

**Survival of Lymphocytes in the Circulation**

Figure 2 illustrates the mean percentage of cells (±1 SD) remaining in the circulation plotted on a logarithmic scale at the times indicated on a linear scale. Both normal subjects and patients with CLL show a rapid clearance of the cells from the circulation and at least two exponential clearance rates can be extracted from the data for the initial 4-hr period. For the normal subjects the clearance rates have half times of 15 min and 7 hr, as indicated in the figure.

An interesting difference is noted between the patient with CLL and normal subjects in this initial period in that approximately 20% of the injected activity remains in the circulation at 4 hr in the patients with CLL, whereas only 2% of the total remains in the circulation of the normal subjects. This result is in accord with the postulate made by other workers that lymphocytes in CLL subjects do not leave the circulation as do normal lymphocytes. The percentage of cells remaining in the circulation in the initial period of the study is

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Fig. 2.—Survival of autotransfused lymphocytes in human subjects. Closed circles, and solid lines, normal subjects; closed squares and solid lines, subjects with chronic lymphatic leukemia; open circles and solid lines, heat-killed cells.
similar to that found in CLL patients by Stryckmans et al.\textsuperscript{17} and Fliedner\textsuperscript{18}

After the initial clearance phase a more gradual clearance of the cells from the circulation is seen and the half-times for residence in the circulation is seen to be 1.7 days for the normal subjects and 5 days for the CLL patients. This latter half-time is similar to the half-time of 5.6 days found by Pfisterer et al.\textsuperscript{8} in CLL patients. Calculation of mean duration in the circulation on the basis of an exponential clearance rate from the circulation gives times of 2.5 days and 7.5 days, respectively, for the normal subjects and CLL patients. (Counting of blood samples was discontinued when the counts obtained from the washed cells from the 10-ml blood specimens fell to half the background counting rate, since the counting error at this level becomes too large for any significance to be attached to the results obtained.\textsuperscript{19} All normal subjects to the 4-day period had counts in excess of twice the background level but after this period most had counts at or below this level.) After the 8-day period insufficient subjects remained in the study to chart a group survival, but counts in excess of the background at levels of 0.2–0.3% of the injected dose were recorded in two subjects at 10 and 12 days. No sufficiently similar study has been made to compare these results with, although the study by Pfisterer et al.\textsuperscript{8} does show levels in the circulation of 0.1–0.5% over the 13–48-day period. It is possible in this latter study that radioactivity attached to the lysed red cells may account for the slightly higher values recorded.

Surface Studies

Figure 3 illustrates the surface activity recorded over the spleen, liver, and bone marrow (iliac crest) for 10 normal subjects, four patients with chronic lymphatic leukemia, and three control subjects injected with heat-killed cells.

Several points of interest arise from these surface studies. In terms of uptake over these sites a fourfold uptake above the initial 100-sec reading is noted over the spleen and iliac crest in the normal subjects and a twofold uptake over the liver. The time at which the maximum uptake occurs is noted to be 2–3 hr over the spleen, 30 min over the liver and longer than 4 hr over the iliac crest. (Not included in Fig. 3 is the activity recorded over the central abdomen designed to study the uptake in lymphatic time within the intestine and mesenteric lymph nodes. It was found that the pattern of activity recorded over this site was similar to that over the iliac crest, which would seem to indicate that either similar tissues are being studied in both sites, such as bone marrow or lymphatic tissue, or that both bone marrow and lymphatic tissue have similar patterns of activity.)

There is very little published work with which to compare these results but the pattern is clearly different from those of Duvall and Perry,\textsuperscript{7} where a mixed population of leukocytes were labeled and from those of MacMillan and Scott\textsuperscript{6} using a similar mixed leukocyte suspension. In the experimental work in rats, modal transit times of 5–6 hr for the spleen and 24 hr for the lymph nodes have been found. The present study shows some similarity to
these studies in that the times of peak uptake over the spleen and sites overlying lymphatic tissue follow this general pattern.

Another point of interest in these surface studies is the difference in pattern obtained for the CLL patients. Over the iliac crest and abdomen, virtually no uptake is seen until 24 hr, and a peak twofold uptake is seen at 48 hr. In the liver a different pattern is also observed in that there appears to be a more rapid release of the leukemic cells from this site. The actual reasons for the observed differences in the pattern obtained in the CLL patients is uncertain, but to the extent that the pattern is a consistent one in the four patients studied, it would appear to be due to an intrinsic abnormality in the leukemic cell resulting in defective migration to and from the circulation.

In regard to the surface pattern obtained in subjects injected with heat-killed intact cells, the results are important in assessing the significance that
can be attached to the surface studies. Over the spleen, iliac crest, and abdomen, a low peak uptake occurring at 24 hr was noted. This pattern is quite different from that obtained after the injection of viable cells into normal subjects and is good evidence that the pattern obtained in normal subjects is not due to passive trapping or reticuloendothelial uptake of these cells, as could be expected to be occurring in the subjects injected with heat-killed cells. Over the liver a similar proportion of the injected dose is taken up as when viable cells are injected, although the rate is somewhat slower. This result is the same as that found in mice by Bainbridge et al.2 after the injection of viable and heat-killed lymphocytes in these animals.

The activity recorded over the lungs in normal subjects in all cases shows a rapid exponential falloff of activity in the initial period of study, which contrasted with the pattern obtained over the lungs in subjects injected with heat-killed cells. Here it was noted that the activity was slightly higher and fell off at a slower rate, suggesting that the damaged cells, unlike the normal lymphocytes, were trapped in the lungs. Very little difference was noted between the normal subjects and the CLL patients. The activity recorded over the heart showed a rapid exponential decline that parallels the decline occurring in the bloodstream.

**DISCUSSION**

One of the major difficulties in a study of this sort is to assess the viability of the labeled cells within the body, as it is clear that the significance that can be attached to the studies is dependent upon the cells’ being viable and functionally unaltered by the handling procedure in vitro.

The evidence put forward in this work that the cells are viable depends on (1) the markedly different surface studies and blood clearance curves observed between the viable and heat-killed intact cells and (2) the assessment of 24-hr excretion rate of $^{51}$Cr in the urine. The validity of this latter measure of in vivo viability depends upon the evidence put forward in this study and also in experimental work in animals$^{2,29}$ that when labeled cells are killed in vitro and both the cell debris and released $^{51}$Cr is injected i.v., approximately 60% of the radioactivity is excreted in the urine within 24 hr. On this basis it is possible to derive a crude quantitative estimate of cell life using the urinary $^{51}$Cr excretion data, in that the amount excreted can be regarded as representative of 60% of the cells that die within the body during the period of urine collection. (For example, applying this estimate to the data in Table 1 for normal subjects would give the percentage of cells dying over the 8-day period as 28%, which also correlates with the rate of decay observed over such sites as the spleen and iliac crest. In terms of half-life, a value of 17 days can be derived from the data, assuming exponential rates of cell death. The main errors in such estimates result because some of the $^{51}$Cr is that eluted from viable cells and some, after the first 24 hr, is from $^{51}$Cr bound to the body tissues. However, in both cases the error results in an underestimate of cell death, and urinary excretion rates can be regarded as an estimate of minimal life-span of the cell.)

Several of the findings in the study require further comment. One of these
is the apparent short residence time in the circulation noted for the labeled lymphocytes. The survival in the circulation clearly cannot be related to the survival in the body as a whole, as for example, is possible for red blood cells and other estimates of life-span, such as urinary excretion studies, are needed for this purpose. Even so, there are a number of studies suggesting in man and animals that the majority of lymphocytes have a life-span in excess of 100 days and that they can be detected in the circulation over this period.21-23 In view of this evidence from these other sources it is somewhat surprising that a longer half-time of residence within the circulation could not be demonstrated in this study. One explanation that can be put forward is that the labeled lymphocytes consist of two populations of cells, one being a short-lived population that does not migrate from the circulation and the other a long-lived population that does migrate from the circulation. In these circumstances it would be expected that after the initial clearance phase the majority of the long-lived cells would be diluted in the total body pool of lymphocytes and contribute very little to the radioactivity in the small blood samples used in this study. The activity in the blood then becomes predominantly due to the short-lived nonmigratory population and the short half-time observed within the circulation would be of this selected population.

The mean life of 2.5 days is in fact similar to the population found by Otteson21 to have a life-span of 3 days. Moreover, there is some evidence in animal experiments24 that short-lived lymphocytes do not migrate from the circulation as do small long-lived lymphocytes, so this explanation has some support. Presumably to demonstrate the long-lived lymphocytes within the circulation, larger blood samples need to be taken to obtain sufficient radioactivity for counting purposes.

Another consideration arising from this study is to what extent a mathematical analysis of the data is possible similar to the established analysis of distribution of a label in a multicompartmental system.19 Observation of the data suggests that there is an interrelationship between the various sites under study. For example, in the initial period of the study the rapid clearance phase from the circulation can be correlated with the rapid uptake of the cells into the liver and spleen and the slower clearance phase can be correlated with the more gradual uptake occurring into the sites recorded by probes over the iliac crest and abdomen. In the CLL patients the second clearance phase of the initial period of study is seen to be much slower and this can be correlated with the much slower uptake of lymphocytes noted over the iliac crest and bone marrow sites.

There are several obvious difficulties in attempting an analysis of this sort. One of these is the lack of data from all regions of the body likely to be involved in the cell transfer from one compartment to another. Also, allowance has to be made for such organs as the liver, where it is likely that the activity recorded reflects the activity of two types of tissue, such as the reticuloendothelial tissue trapping damaged cells and the tissue responsible for the normal uptake of lymphocytes shown to occur in the sheep.1 Quantitation of the changes observed under the probes is also difficult and, at best, gives only estimates of the quantitative events occurring.
Despite these objections it is useful to attempt such an analysis in that some insight into the factors important in this interchange of cells between compartments is gained. The mathematical model found that gives the best interpretation of the data is that derived for an open multicompartmental mamillary system, which is described by the mathematical expression

\[ f(t) = A(1 - e^{-Bt}) \]

where \( A \) is the value at the equilibrium point and \( B \) is the rate constant of return of cells from the site studied. It can be shown mathematically that for the model under discussion the expression becomes:

\[ f(t) = C_0 e^{-k_1 t} \left( \frac{k_2}{k_2 + k_3} \right) \left( 1 - e^{-(k_2 + k_3)t} \right) \]

From this it is seen that the value of \( A \), the equilibrium point, is determined by the concentration of labeled cells in the circulation (which is described by the expression \( C_0 e^{-k_1 t} \), \( C_0 \) being the concentration of cells at time zero and \( k_1 \) the sum of the exponential clearance rates from the circulation) and the rate of uptake \( k_2 \) and release \( k_3 \) from the site under study. The value of \( e^{-Bt} \) is seen to be determined by the sum of the rate of uptake and release from the site and it is this value that determines the rapidity with which the equilibrium point or the time at which maximum uptake is reached. In terms of the same expression and if there is a continuing free exchange of cells between the organ and the total body pool of lymphocytes, it can be shown that the decline in activity in an organ after the point of maximum uptake can be described by substituting \( N_0 e^{-k_1 t} \) for \( C_0 e^{-k_1 t} \) in the above expression. \( N_0 \) in this case is the number of cells in the organ at the time of maximum uptake and \( k_1 \) in this case is the death rate for the cells in the body as a whole. (This in turn can be related to the rates of excretion of \(^{51}\)Cr in the urine.)

Similarly, the clearance curve from the circulation can be described by the standard expression for a curve resulting from multiple exponential clearance rates:

\[ C(t) = ae^{-k_1 t} + be^{k_2 t} + \ldots \]

where \( C \) is the number of cells remaining in the circulation at time \( t \) and \( k_1, k_2, \ldots, \) the rates of clearance into the various compartments. Given sufficient data it should be possible to relate these clearance rates to individual rates of uptake of the organs concerned.

In conclusion, the separation and labeling of lymphocytes with \(^{51}\)Cr using the techniques outlined has proved to be relatively simple and reproducible procedure. The in vivo studies expose the subjects to no more risk or discomfort than equivalent in vivo radionuclide tests, such as platelet survival studies or iron kinetic studies, and seem applicable on a clinical research basis. Using these techniques it is possible to detect differences between normal and abnormal states and the results can be subjected to mathematical analysis, which it is hoped will enable more precise measurements of lymphocytes within the body.

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