Neutrophil Preservation

THE EFFECT OF SHORT-TERM STORAGE ON IN VIVO KINETICS

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ABSTRACT A rabbit model was developed to study the in vivo viability of neutrophils stored in vitro for up to 72 h. Acid-citrate-dextrose anticoagulated whole blood was obtained from rabbits previously injected with tritiated thymidine ([^{3}H]thymidine), stored under varying conditions, and then injected into recipient rabbits. Neutrophil viability and function were assessed by measuring the ability of the tagged neutrophils to circulate and to migrate into subcutaneous polyvinyl sponges.

Unstored neutrophils disappeared exponentially from the circulation with a $t_{\frac{1}{2}}$ of 3.2 h and gave a zero time recovery of 30.5%. Storage of cells at either room temperature or 4°C for 24 h or longer resulted in temporary sequestration of cells from active circulation. With cells stored for up to 72 h at 4°C, recovery returned to normal values after 1–2 h. Room temperature stored cells, in contrast, showed evidence of irreversible damage at 48-h storage with low recovery for the entire time span studied.

With unstored blood, 8.1±0.9% of the injected neutrophil label was present in the subcutaneous sponges. The accumulated label progressively decreased as cell storage time increased reaching at 72 h 5.1±0.6 and 2.6±0.3% for 4°C and room temperature-stored cells, respectively.

The results of this study indicate that 4°C storage of rabbit neutrophils is superior to storage at room temperature. The data suggest that it may be feasible to store neutrophils at least a few days without loss of in vivo functions.

This work was presented in part at the Annual Meeting of the American Society of Hematology, 1975. *Blood.* **46:** 1055. (Abstr.)

Received for publication 30 August 1976 and in revised form 9 November 1976.

INTRODUCTION

It has generally been thought that polymorphonuclear leukocytes (PMNs)[1] rapidly lose their capacity to circulate in vivo after being stored in vitro for more than a few hours (1, 2). As a result, most centers use only freshly collected PMNs for clinical granulocyte transfusions. PMN transfusion programs would be greatly facilitated if it were possible to maintain in vivo function during in vitro storage. Recent data indicate that PMNs may be stored for 24 h without significant impairment of certain in vitro functional capabilities (3, 4). These findings, plus our concern for optimizing the availability of PMNs for transfusion, prompted us to reexamine the effects of in vitro storage on the in vivo kinetics of PMNs. For these studies an animal model was developed to study the blood kinetics and tissue localization of PMNs subjected to various storage conditions.

METHODS

General experimental procedure. New Zealand rabbits of either sex weighing approximately 3 kg were used for all experiments. One group of animals was used to measure the blood kinetics of stored PMNs; a separate group was used to measure the localization of stored PMNs at inflammatory sites.

Blood PMNs were labeled by injecting donor rabbits intravenously with 2 mCi of [^{3}H]thymidine (40–60 Ci/mM; New England Nuclear, Boston, Mass.), as in previous PMN kinetic studies from this laboratory (5). The time course of the labeling of circulating PMNs was determined in three animals (Fig. 1), PMN specific activity being determined as described below. 70–72 h after injection of the isotope, blood containing labeled PMNs was drawn from the unanes-

[1] *Abbreviations used in this paper*: ACD, acid-citrate-dextrose; PMN(s), polymorphonuclear leukocyte(s); RT, room temperature.

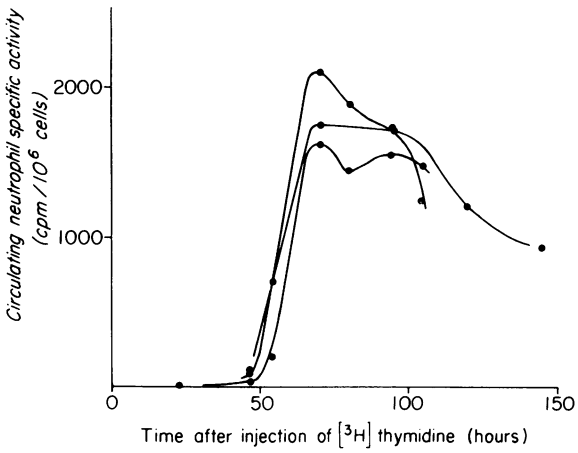

FIGURE 1 Tritium activity in circulating PMNs after the intravenous injection of 2 mCi of [^{3}H]thymidine. Each line represents the data on one animal.

thetized donor animal by cardiac puncture into a plastic syringe using acid-citrate-dextrose (ACD) as the anticoagulant. This tagged blood was stored up to 72 h without agitation in the plastic syringe at either room temperature (18–20°C) (RT) or at 4°C. It was then injected intravenously into normal recipient animals to study either the blood kinetics of the stored PMNs or the ability of the stored PMNs to accumulate at an inflammatory site.

For measurement of the blood kinetics of stored PMNs, 10 ml of stored blood was injected into the marginal ear vein of the recipient animal over a 2-min period. The midpoint of the injection was considered time zero. 6-ml blood samples were obtained at 10, 60, 150, 240, 330, and 420 min to determine the rate of disappearance of the labeled PMNs. Blood samples were obtained by slashing the marginal vein of the ear, via an ear vein polyethylene catheter, or by cardiac puncture. Recipient animals were used only once to avoid problems with isosensitization.

The ability of the stored PMNs to localize at an inflammatory site was studied in vivo by measuring PMN accumulation in subcutaneously implanted polyvinyl sponges, as has been previously described (6). Polyvinyl sponges packed in formalin (Unipoint Corporation, High Point, N. C.) were cut into 3 × 3 × ⅓ cm pieces, washed 20–30 times in tap water, sterilized by boiling 10 min in distilled water, and soaked in sterile saline before use. Rabbits were tranquilized with 3 mg acepromazine maleate and anesthetized with pentobarbital. After shaving and cleansing the skin of the back with iodine and alcohol, a 3–4 cm midline incision was made to the level of the subcutaneous fascial space. The sponges were removed from the saline, blotted dry with sterile gauze, and six sponges were inserted into the subcutaneous tissue of each rabbit, three on each side. The sponges were placed flat and at least 1 cm lateral to the incision. The incision was closed with sutures or metal clips and sealed with collodion. To augment cell migration to the sponges, 0.2 ml of a saline solution of *Salmonella typhosa* endotoxin (Difco Labs., Detroit, Mich.) was added to the sponges in most experiments before insertion. The stored blood samples containing [^{3}H]thymidine-labeled PMNs were injected into the sponge-laden rabbits as for the blood kinetic studies. The animals were later sacrificed by intracardiac injection of a barbiturate solution, the incision reopened, and the sponges removed. The accumulation of stored PMNs in the sponges was determined by measuring the sponge tritium activity. To assess the degree to which plasma radioactivity was contributing to activity in the sponges, plasma prepared from labeled fresh blood was injected into sponge-laden recipient animals and sponge tritium activity measured.

PMN separation. PMNs were isolated by Hypaque-Ficoll sedimentation (7). 6 ml of ACD-anticoagulated blood was carefully layered over 3 ml of a mixture of 10 parts 33.9% Hypaque (sodium diatrizoate U.S.P., Winthrop Laboratories, New York) and 24 parts 9% Ficoll (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) (specific gravity of 1.077–1.078) in 13 × 150-mm plastic tubes (Falcon Plastics, Division of Bio Quest, Oxnard, Calif.). The sample was spun at 400 *g* for 35 min at RT. The supernate and cells above the erythrocyte layer and the top 2–3 mm of the erythrocyte layer were discarded. 6 ml of 3% dextran (mol wt 200,000–300,000, I.C.N. Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio) in 0.9% saline was then added and the contents thoroughly mixed. The tubes were placed at a 45° angle until a distinct demarcation between the erythrocyte layer and the PMN-rich layer occurred. The PMN layer was removed and serial washes with 5–8 ml of 0.87% NH_4Cl were used to lyse the remaining erythrocytes. After washing until no trace of red color remained, the final pellet was suspended in 12 ml of 0.87% NH_4Cl, and an aliquot was removed for cell count and smear. Cell counts were performed with a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.). Smears made from such preparations revealed no erythrocytes or platelets. In 55 such isolations the percentage of PMNs varied from 96 to 100% (mean 98.7%). The remaining cells were pelleted and solubilized in 1 ml NCS (NCS tissue solubilizer, Amersham/Searle Corp., Arlington Heights, Ill.). 16 ml of scintillation fluid (1,000 ml toluene, 5 g PPO, and 0.36 mg dimethyl POPOP, Packard Instrument Co., Inc., Downers Grove, Ill.) was added and the sample counted in a liquid scintillation counter (Packard Instrument Co., Inc.). Activity was expressed as counts per minute per 10^6 cells. PMN yield from the original blood samples was generally 30–50%.

Labeling of other blood components. The relative labeling of the components of the unstored donor blood (Table I) was determined by fractionating it in the following manner. Plasma was obtained by centrifuging an aliquot of the sample at 2,000 *g* for 10 min. Platelet-rich plasma was obtained by spinning the labeled whole blood at 100 *g* for 10 min; the contaminating leukocytes were sedimented by spinning the supernate at 500 *g* for 5 min. The platelets were washed three times in saline and platelet buttons prepared. A suspension of mononuclear cells was obtained by aspiration of the upper white layer from a Hypaque-Ficoll

TABLE I
Relative Labeling of Blood Components 3 Days after [^{3}H]Thymidine Injection

	Specific activity	Relative activity
	cpm/10⁶ cells	*per ml blood*
Neutrophils	790	1
Mononuclear	198	0.5
Erythrocytes	<0.25	<0.9
Platelets	0.18	0.03
Plasma	—	45

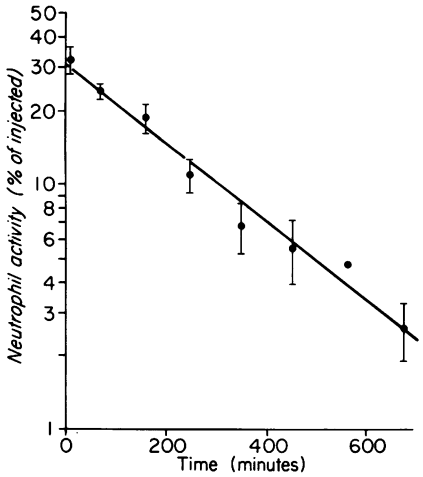

FIGURE 2 Disappearance of neutrophil radioactivity after intravenous injection of fresh [^{3}H]thymidine-labeled rabbit PMNs (14 animals). Vertical lines represent 1 SEM.

cell separation. These cells were washed three times with saline and the mononuclear cells pelleted. PMN pellets were prepared as described above. Erythrocytes were prepared by sedimenting the neutrophil-red cell fraction after Hypaque-Ficoll sedimentation twice in 3% dextran and washing the erythrocyte sediment three times with saline. To equalize quenching, each sample was solubilized in 100 μl of 60% $HClO_4$ and 200 μl of 30% H_2O_2 by digestion for 60 min in an 80°C water bath. 5 ml of 2-ethyoxyethanol and 10 ml of scintillation fluid were added (8). Tritium activity in these samples was 790 cpm/10^6 PMNs, 198 cpm/10^6 mononuclear cells (corrected for contaminating platelets), 0.18 cpm/10^6 platelets, and 97,000 cpm/ml plasma. Erythrocyte activity was 0.25 cpm/10^6 cells, but this must be considered a maximum since there was enough contamination of this fraction with PMNs to account for all the label seen. Based on these findings, less than 1% of the label in the average PMN isolate was derived from non-neutrophilic cells.

Processing of sponges. The cells in the removed sponges were isolated by rinsing the sponges two to three times in calcium-magnesium free Hank's solution made 1 mmol in EDTA, the cell suspension being expressed from the sponge by manual squeezing. The cell suspension was incubated at 37°C for 30 min with pronase (1 mg/ml) to reduce leukocyte clumping. Cell counting was performed both manually and with a Coulter counter and total leukocyte count/sponge was determined. Differential counts on the cellular suspension revealed the cells to be only PMNs. Microscopic section of the sponges before and after squeezing confirmed that after squeezing only a small number of cellular elements remained, an observation that has been noted by other investigators (6). For determination of tritium activity the sponges were air dried, wrapped in filter paper, and compressed into a pellet with a pelletizer (Parr Instrument Co., Moline, Ill.). The pellets were burned in a sample oxidizer (Packard Instrument Co., Inc.) and the tritium content determined by liquid scintillation counting with Oxifluor (New England Nuclear) as the scintillant.

Calculations. For calculation of percent PMN recovery, rabbit blood volume was assumed to be 50 ml/kg. PMN specific activity and PMN counts per minute per milliliter of blood were plotted versus time on semilog graph paper. Lines were drawn through the linear portions of each curve by the method of least squares. Extrapolated PMN recovery was calculated in standard fashion (2) from the values obtained by extrapolating this line to time zero. Circulating PMN activity at each time point was then expressed as a percent of the extrapolated value so that values for each time could be pooled and averaged for all animals representing a given storage condition. From this data composite curves were constructed for each storage condition relating percent PMN recovery to time.

RESULTS

Normal kinetics of the model. The blood kinetics of unstored PMNs were measured in 14 animals (Fig. 2). PMN disappearance from the circulation was exponential suggesting random removal of cells from the circulation as in previous studies (2, 9–11). PMN recovery (the percent of injected PMN label accounted for in the circulation) extrapolated to zero time was 30.5%, a value somewhat lower than seen in other species (2, 9, 10). The $t_{\frac{1}{2}}$ disappearance time was 3.2 h, also a smaller value than reported in other species (2, 9–11).

The migration of PMNs into the subcutaneous sponges as a reflection of time after sponge placement is shown in Fig. 3. PMN accumulation plateaued at 12–18 h at 30×10^6 cells/animal. In an attempt to increase the number of cells, 0.2 ml of a saline solution of varying amounts of *S. typhosa* endotoxin was added to each sponge. Cellular accumulation at various endotoxin concentrations was assessed at 18–24 h (Fig. 4). Because endotoxin so greatly in-

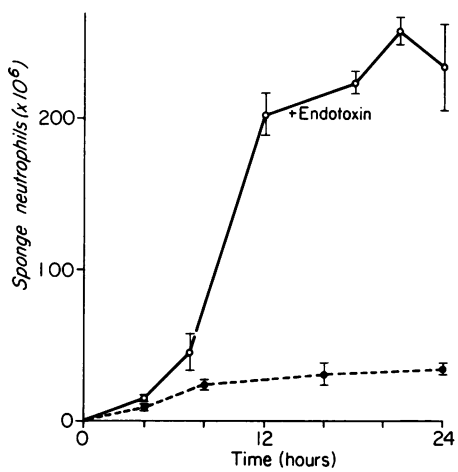

FIGURE 3 The accumulation of PMNs in subcutaneously implanted polyvinyl sponges. Solid circles represent the number of PMNs accumulating per animal in sponges implanted without endotoxin; open circles represent PMNs accumulating per animal in sponges with 1 mg *S. typhosa* endotoxin per sponge. The vertical lines at each point represent 1 SEM. Each point represents data on three to eight animals.

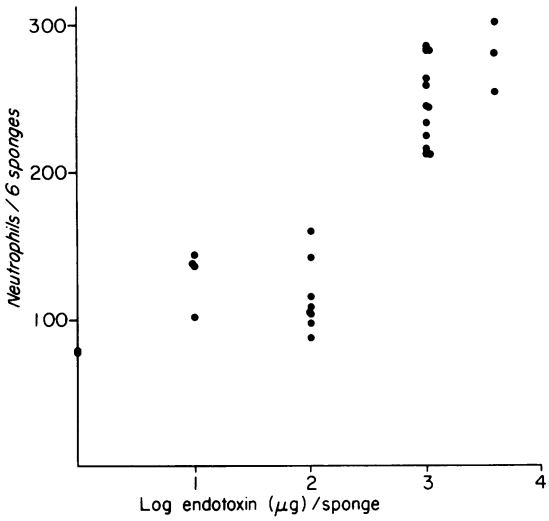

FIGURE 4 Effect of increasing amounts of endotoxin on the number of PMNs accumulating in sponges 18–24 h after implantation. Each point represents one experimental animal.

creased the cellular accumulation and caused no apparent adverse effects in the rabbits, in subsequent experiments 1 mg of endotoxin in 0.2 ml of saline was added to each sponge before insertion into the animal. The time course of PMN accumulation was determined at this standard dose of endotoxin (Fig. 3). Cellular accumulation was minimal for the first 4 h, then increased dramatically and plateaued at 18–24 h at levels of 200–250 × 10^6 cells/animal. To evaluate the ability of stored PMNs to localize in these sponges, the stored blood samples containing [^{3}H]thymidine-labeled PMNs were injected into rabbits into which sponges had been inserted 4 h previously. 20 h later (24 h after sponge placement) the sponges were removed and their tritium activity was determined. Tritium activity recoverable from the sponges was expressed as a percent of the injected PMN radioactivity. The sponge kinetics of unstored [^{3}H]thymidine-labeled PMNs was studied in nine animals. With these unstored cells, 8.1±0.9% of the injected PMN label was present in the six sponges 20 h after injection. Some accumulation of activity in the sponges occurred after injection of plasma prepared from labeled blood. In three recipients an average of 0.033% of the injected plasma label was recoverable in the sponges. Since plasma label per milliliter of blood averaged 45 times the PMN label, it can be estimated that about 1.5% of the presumed PMN activity in the sponges was derived from plasma. This has the effect of making the base line of Fig. 6 approximately 1.5%.

The effect of storage time and temperature on blood kinetics. The kinetics of cells stored at RT for up to 48 h are shown in Fig. 5A. Percent recovery and $t_{\frac{1}{2}}$ were normal through 8 h of storage. Beginning possibly at 18 h, and certainly by 24 h, the initial recovery of the cells (that is, recovery as measured at the 10-min sample) decreased with increasing storage time, reaching a level of 10.2% at the 48-h storage. Of particular note, with storage times of 18–24 h or

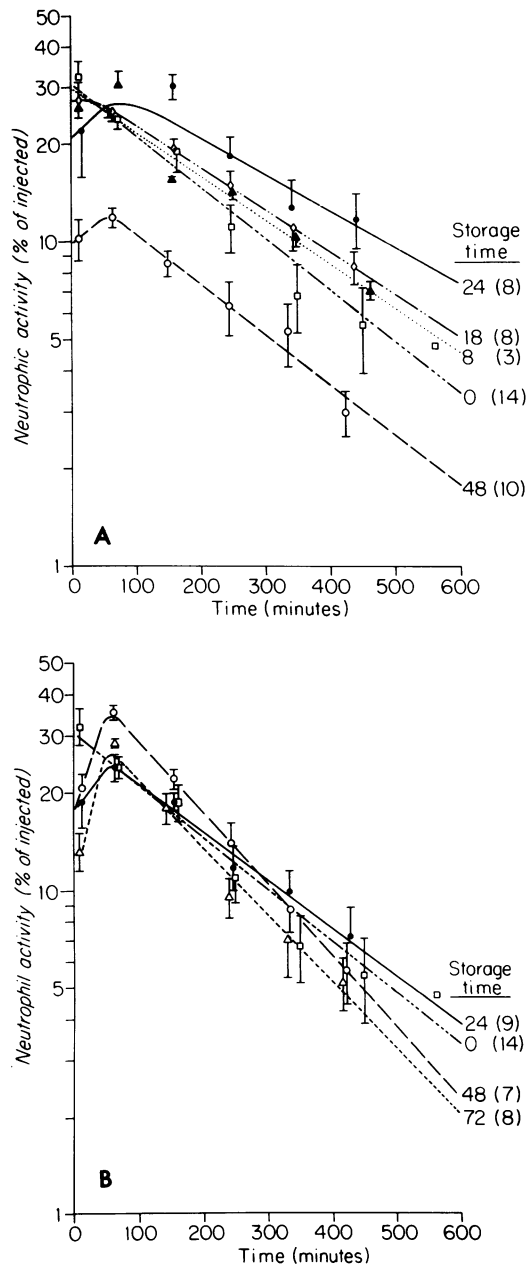

FIGURE 5 Disappearance of neutrophil radioactivity after intravenous injection of stored [^{3}H]thymidine-labeled rabbit PMNs. Storage time (hours) is indicated at the right of each curve. The number of animals studied at each time is indicated in parentheses. The symbols for the storage times are: □ zero h; ▲ 8 h; ◇ 18 h; ● 24 h; ○ 48 h; △ 72 h. (A) Blood stored at RT; (B) Blood stored at 4°C.

longer, the specific activity disappearance curves were no longer linear. After initial low recovery, the number of circulating labeled PMNs increased to a maximum at 1–3 h, after which it decreased linearly, the $t_{\frac{1}{2}}$ of the linear portion of the curve being the same as for fresh cells. Through 24 h of storage the percent of injected cells circulating by 2 h was not significantly different from normal. However, with cells stored for 48 h, although there was increasing recovery for the first 1–2 h, recovery remained below the normal curve for the entire study.

The kinetics of PMNs stored at 4°C are shown in Fig. 5B. As with RT-stored cells, initial recovery decreased with increasing storage time, reaching a level of 13.2% at 72 h. With storage times of 24 h or greater, there was increasing recovery after the initial sample, recovery being maximal after 2 h and falling off linearly thereafter. In contrast to cells stored at RT, cells stored at 4°C for as long as 72 h showed normal recovery by 2 h, and the disappearance curves were subsequently not distinguishable from normal. The $t_{\frac{1}{2}}$ of the linear portion of the curves remained relatively constant at either RT or 4°C storage through the storage times studied.

Effect of storage time and temperature on in vivo PMN localization. 63 recipient animals were studied with tagged blood stored for up to 72 h at either RT or 4°C. The results are shown in Fig. 6. As mentioned previously, with unstored cells, 8.1±0.9% of the injected PMN label was present in the six sponges 20 h after injection. The ability of the cells to localize in the sponges decreased with storage time, reaching 2.6 ±0.3% at 72 h for RT-stored blood and 5.1±0.6% at 72 h for 4°C-stored blood.

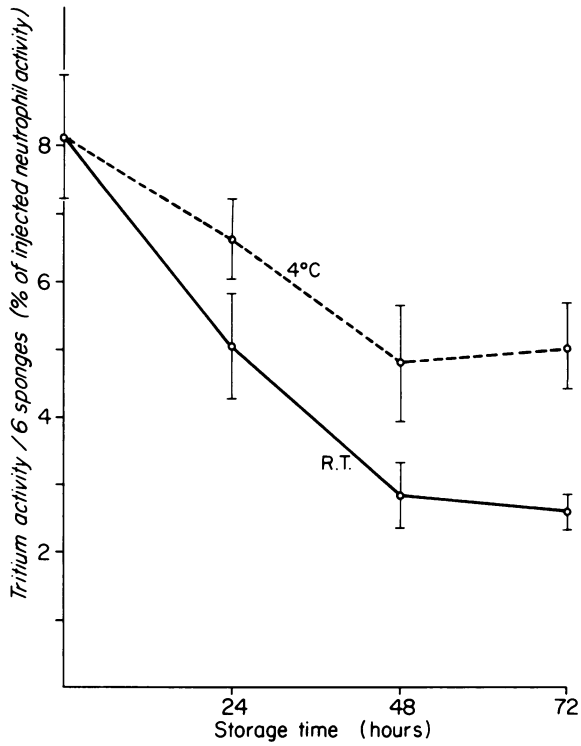

FIGURE 6 Effect of storage time and temperature on the amount of tritium activity accumulating in subcutaneous sponges. Stored [^{3}H]thymidine-labeled PMNs were injected intravenously 4 h after sponge placement. Sponges were removed 20 h later. Sponge tritium activity per animal is expressed as percent of injected neutrophil label. The solid line represents RT storage. The dotted line represents storage at 4°C. Vertical lines at each time point represent 1 SEM. Each point represents data on 6–12 animals.

DISCUSSION

Previous studies on PMN preservation have primarily dealt with attempts at long-term storage with cryopreservation techniques (12–14). Researchers have taken this approach because PMNs have generally been presumed to retain viability in vitro for only a matter of hours. Recent studies suggest that this may not be the case. McCullough et al. (3, 4), investigating the effect of time and anticoagulant on the viability of human PMNs with a variety of in vitro function tests, showed that in vitro bactericidal function and the ability of the PMNs to reduce nitroblue tetrazolium remained normal in cells stored up to 24 hours at 4°C in ACD or citrate-phosphate-dextrose. Chemotactic activity of the cells was only minimally impaired after similar 24-h storage. With storage times longer than 24 h, findings have been variable depending on the function investigated. Bactericidal activity was retained through 72 h of storage, but nitroblue tetrazolium reduction and chemotaxis of the cells were progressively impaired. Since there is some evidence that these changes are reversible in vivo (15, 16), their interpretation is difficult.

The in vivo function of PMNs may be divided into three parts: the ability of the cells to circulate normally, the ability of the cells to localize at areas of inflammation, and the ability of the cells to carry out phagocytic and bactericidal activity at the inflammatory site. In these studies we have attempted to evaluate the first and second of these functions.

The kinetic data indicate that rabbit PMNs can be stored for up to 3 days without significant loss of their ability to circulate. With storage at either RT or 4°C for 24 h or longer, a portion of the injected labeled cells is temporarily sequestered from active circulation. These cells return by 2 h and are thereafter lost from the circulation at a normal rate. There appears to be a cell defect that occurs with the conditions of storage used in this study (ACD anticoagulant; stationary storage in whole blood) which is reparable in vivo by 2 h time. Since all the survival curves have reached a maximum recovery by 2 h after injection of the labeled cells, it seems reasonable

to consider variation from normal 2-h recovery as a measure of irreversible PMN damage. On this basis, cells stored at 4°C show no permanent cell damage through 72 h of storage. In contrast, cells stored longer than 24 h at RT have sustained irreversible damage as reflected in reduced recovery at all time points. This would suggest that PMN viability is better maintained at 4°C than at RT but further temperature studies are necessary to determine if 4°C is indeed optimal.

The nature of both the reversible and irreversible PMN defects which occur with in vitro storage is unknown, as is the site of apparent sequestration of labeled cells in the early part of the survival curve. Transient sequestration of platelets damaged by EDTA has been observed and localized to the liver and spleen (17). The exact fate of effete or damaged PMNs has never been elucidated. Interestingly, the cellular defect observed with storage of PMNs was reflected in changes in percent recovery, the $t_{\frac{1}{2}}$ disappearance time of stored cells remaining remarkably constant, an observation also made in early PMN labeling studies (1, 2).

The superiority of 4°C storage over RT storage was also observed for extravascular PMN accumulation in the subcutaneous sponges, but a defect in this aspect of PMN function occurred with both storage temperatures. With unstored blood the sponges accumulated 8.1 ± 0.9% of the injected PMN label. There was a progressive decrease in the percentage of injected label recoverable from the sponges with increasing storage time for both temperatures, reaching a level of 5.1±0.6% and 2.6±0.3% at 72 h of storage for 4°C- and RT-stored cells, respectively. The relative specific activities of the erythrocytes, platelets, and PMNs in tagged blood, in addition to the microscopic absence of mononuclear cells in the sponge fluid, indicate that PMNs are the only cellular element contributing significantly to sponge radioactivity.

This animal model is useful for assessing the in vivo function of stored PMNs. These studies demonstrate that rabbit PMNs stored in whole blood for 72 h at 4°C showed no permanent impairment in their ability to circulate and only a moderate impairment in their ability to localize in vivo at sites of inflammation. Storage for 48 h or longer at RT resulted in significant impairment by both criteria. These observations suggest that it may be possible to store granulocytes for at least 1–2 days for transfusion. If human PMNs can also be stored for this period, it should become substantially easier to provide granulocytes for transfusion in man.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Loretta Willis and Maribeth Spencer.

This work was supported by U. S. Public Health Service Contract NHLI-HB-74-11, National Institutes of Health grant HL-06242, and by the Blood Research and Demonstration Center grant HL-17265.

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