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KINETICS OF NEUTROPHIL-RELEASING ACTIVITY OF POST-LEUKOPHERESIS PLASMA

by

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INTRODUCTION

The studies carried out during the first year of this contract were concerned principally with determining the optimal dosage of plasma from rats which had undergone filtration leukopheresis (PPP) to stimulate a granulocytosis in other rats to be used as granulocyte donors (1,2). The objective of using PPP in granulocyte donors is to increase the harvest of granulocytes from these donors. Ultimately, it is our intention to determine whether stimulation of granulocytosis by this means will be useful in a clinical setting.

We also investigated the effect of duration of pheresis on the granulocyte donor and, subsequently, the degree to which the duration of pheresis affected the response of the recipients to injection of PPP (2).

These studies essentially involved practical considerations of dosage requirements preliminary to initiation of clinical trials of post-pheresis plasma, as a novel and physiological approach to improving granulocyte yields to provide transfusions into septic granulocytopenic recipients.

Basic information on the kinetics of granulocyte mobilization from tissue reserves (marginating granulocyte pool and bone marrow) was also obtained.

During the period of the present task (1 February 1977 to 31 January 1978), these studies have been extended to include the following:

1. Stability of neutrophil-releasing activity of plasma from leukopheresed rats after storage at 4°C (in collaboration with Dr. C. Robert Valeri).

2. Further studies of mobilization of granulocytes on successive days with plasma from leukopheresed rats.

3. Determination of the duration of the granulocyte increment in the recipient following injection of PPP.

4. Initiation of collaborative studies with Dr. Fabian Lionetti, using techniques of radioautography, on the fate of transfused fresh or frozen granulocytes obtained by

    a. filtration leukopheresis
    b. elutriation

5. Initiation of collaborative studies with Dr. Fabian Lionetti on viability of filtered granulocytes, as measured by size change, inclusion of fluorescein diacetate into the cytoplasm and exclusion of ethidium bromide from the nucleus of filtered granulocytes.

6. Comparison of intravenous and intraperitoneal administration of PPP for the induction of granulocytosis in appropriate recipients.

7. Initiation of studies of granulocyte yields in a previously developed rat model using 50% plasma/saline rather than 100% plasma.

8. Determination of the difference between the use of previously used and fresh plasma/saline to elute granulocytes from nylon filters.

9. Initiation of studies to determine the optimal time to pre-treat rat granulocyte donors with PPP.

METHODOLOGY

Stability of Granulocyte-Releasing Activity of Plasma from Leukopheresed Rats after Storage at 4°C

Homologous rat post-pheresis plasma obtained from donors after filtration leukopheresis can be used to increase the granulocyte yields from subsequent donors. For routine clinical use, plasma containing neutrophil-releasing factor (NRF) must be stored for at least 24 hours. Our previous investigations showed that rat NRF is stable for up to two weeks when frozen at -150°C (3). Human NRF is apparently not stable after freezing but may be stable in the non-frozen state (4-6). Our present study was carried out to determine whether rat NRF in post-pheresis plasma is stable for up to three weeks at 4°C.

Sprague-Dawley rats weighing 350 to 380 grams were leukopheresed as described previously (7). Following pheresis, the rats were exsanguinated and the plasma was separated by centrifugation. The plasma was separated into two aliquots; the first to be used immediately and the second to be used after 1, 7, 14 and 21 days of storage at 4°C.

The granulocyte-releasing activity of the plasma was measured in normal rats by determining the increment in the granulocyte count of the recipients following injection of 1.0 ml/kg of PPP via the exposed femoral vein. Within 30 minutes before administration of the stored plasma, samples from each aliquot were streaked on nutrient agar and blood agar plates and added to thioglycollate tubes to determine bacterial contamination. After injection of the fresh or stored plasma, a WBC count and differential were obtained each hour for three hours. The number of peripheral blood granulocytes was derived from the white blood cell count and the percentage of granulocytes in the differential count.

Further Studies of Mobilization of Granulocytes on Successive Days with Plasma from Leukopheresed Rats

We showed previously (2) that the mean granulocyte increments of rats treated with PPP or controls were not significantly different when treated with aliquots of the identical plasma on successive days. In that study, 18 PPP-treated rats were studied, but only three which received normal plasma. An additional four animals were given normal plasma, for a total of seven control animals. Analyses were carried out on these two groups to determine whether the additional control animals altered the results seen previously, when less than half the present number of controls were used. An additional analysis was carried out to examine whether a residuum of the first day's transfusion could be seen on the second day and, if so, whether this residuum could have affected the degree of granulocytosis-releasing activity of the second transfusion.

Determination of the Duration of the Granulocyte Increment in the Recipient following Injection of PPP

Previous studies of the effect of injection of plasma from leukopheresed rats into normal recipients had been carried out for three hours. To determine the duration of the granulocyte increase these studies were extended to include observations of the granulocyte increment for six hours. As in previous experiments, normal rats were pheresed for two hours. The animals were exsanguinated and the plasma was separated by centrifugation. A volume of 1.5 ml/kg of PPP was injected intravenously into normal rat recipients. Granulocyte counts were obtained before and for six hours after injection.

Comparison of the Ability of Intravenous and Intraperitoneal Administration of PPP to Induce Granulocytosis

Studies are in progress to determine the optimal time of pre-treatment of potential rat granulocyte donors with PPP to obtain the greatest yield of granulocytes. Since the time between injection of the PPP and pheresis could be several hours, depending on the results of these experiments,

there would be an advantage in being able to inject the PPP in unanesthetized animals to eliminate the effect of prolonged anesthesia before initiation of pheresis. This might be accomplished by intraperitoneal administration of PPP in awake animals. The following study was carried out to determine whether i.p. administration would be an appropriate means of administration.

Post-pheresis plasma was obtained by the standard method. Normal, anesthetized rats were injected with 1.5 ml/kg of PPP, either intravenously or intraperitoneally. The same plasma was used for i.v. and i.p. administration in each group of rats. A third group received 1.5 ml/kg of saline as control. WBC and differential counts were obtained before and each hour for three hours after injection. Granulocyte increments were calculated for each animal and the mean granulocyte increment for each time period was calculated.

Granulocyte Yields Using 50% Plasma/Saline

As a preliminary to studies concerned with optimal time of pre-treatment of potential rat granulocyte donors, it was necessary to re-establish the optimal conditions for eluting the granulocytes from the nylon filter column. Previous studies (7) had shown that, ideally, the columns should be eluted with about 40 ml of undiluted normal rat plasma with the pH adjusted to 6.5-6.7 with ACD-A anticoagulant. Herzig had also shown that as little as 20% plasma/80% saline could be used for this purpose. However, the yield of granulocytes was substantially reduced. The use of 100% plasma requires the sacrifice of a large number of animals. The present studies were carried out to determine whether a compromise using 50% plasma and 50% saline would yield sufficient granulocytes to provide satisfactory experiments of PPP pre-treatment of rat donors.

Normal Sprague-Dawley rats were pheresed in the standard manner. While the two-hour pheresis was under way, additional rats were exsanguinated via the abdominal aorta using 20 ml capacity plastic syringes fitted with a 20-gauge hypodermic needle and containing 2 ml of ACD-A anticoagulant. This volume of anticoagulant provides a ratio of one part ACD-A to nine parts of

blood. After sufficient blood was collected, the plasma was separated by centrifugation at 750 x g for 30 minutes. For each filter to be eluted, 20 ml of plasma was mixed with 20 ml of saline and the pH was adjusted to 6.5-6.7 by addition of an appropriate volume of ACD-A.

Preliminary to this study, a single filter was flushed in each of the following ways:

a. Forcing 40 ml of 50% plasma/50% saline through the filter.
b. Flushing with 20 ml of saline to remove contaminating red blood cells prior to flushing with 20 ml of 100% plasma.
c. Gravity flow of 22 ml of saline wash followed by gravity flow of 25 ml of 100% plasma.

Following elution of the granulocytes from the filters by whatever method, the volume, the WBC count, and the differential WBC count of the eluate were determined, and from these, the total number of granulocytes was calculated.

Comparison of the Use of Fresh and Previously Used Plasma/Saline for Eluting Granulocytes from Nylon Fiber Columns

Even the use of 50% plasma, rather than 100% plasma, requires, in the case of animal experiments, sacrifice of a large number of animals to provide the plasma in any study of substantial size. In clinical use, large volumes of human plasma must also be used. Washing of filters obtained from human donors can be done with small chance of contamination because the technique is carried out in a substantially closed system. It would be advantageous both in animal and in human use of filtration leukopheresis if the plasma/saline used for elution of the granulocytes from the filters could be used more than once.

The present studies were designed to determine whether 50% plasma/saline could be used to elute granulocytes from a filter, frozen, and re-used one or more days later.

Plasma was obtained as described previously. It was diluted 1:1 with saline and 40 ml of the diluted plasma was used to elute the granulocytes from the filter. Following calculation of the granulocyte yield, the plasma solution was centrifuged at 750 x g for 30 minutes to remove the granulocytes. The plasma was frozen. On the following day, the plasma/saline was thawed under warm tap water. The pH was re-adjusted to 6.5-6.7. It was used to elute the granulocytes from a filter used in a filtration-leukopheresis process carried out on the same day. The number of granulocytes eluted from the second filter was compared with that obtained from the filter used the previous day.

Determination of the Optimal Time to Pre-treat Rat Granulocyte Donors with PPP

Previous studies by us (8) have shown that pre-treatment of rat granulocyte donors with PPP one hour before filtration leukopheresis results in a substantial increase in the granulocyte harvest. It is possible (and indeed probable) that pre-treatment with PPP at a time other than one hour before pheresis may result in an even greater harvest of granulocytes. The present experiments are being carried out to determine whether this is so.

To this point, only non-stimulated granulocyte donors (controls) have been used. In the process of obtaining post-pheresis plasma for other experiments previously described, the number of granulocytes obtained from the filters used in those studies was determined. The results of these calculations will form part of the basis for the studies involving PPP-stimulated donors.

Viability of Filtered Granulocytes Determined by Dye Exclusion and Inclusion, and by Cell Sizing

This preliminary study was done in collaboration with Dr. Fabian Lionetti at the Blood Research Institute, Boston. Future collaborative studies are planned with Dr. Lionetti to compare the relative viability

of rat granulocytes harvested by filtration leukopheresis and by elutriation. A quick and non-optimal study of the viability of the granulocytes obtained by filtration leukopheresis of the rat (and described above in the section describing three methods of granulocyte elution from the filter) was carried out. After harvesting those granulocytes, they were concentrated by gentle centrifugation. They were incubated with fluorescein diacetate and ethidium bromide according to the method of Dankberg and Persidsky (9). The viability was determined by measuring the inclusion of fluorescein diacetate into the cytoplasm and exclusion of ethidium bromide from the nucleus. An attempt was also made to determine the size distribution of these cells.

Preliminary Studies of the Use of Whole-Body Radioautographic Methods to Determine the Fate of Transfused Granulocytes

A quick and non-optimal study was carried out in collaboration with Dr. Fabian Lionetti to determine the distribution of infused fresh and frozen granulocytes in normal guinea pigs. Fresh guinea pig granulocytes were obtained by Dr. Lionetti with the use of the "Elutriator." The granulocytes were tagged with ^{14}C-DFP. The cells were concentrated. The tagged cells were brought to Arthur D. Little, Inc., and injected into a normal guinea pig via the femoral vein. Thirty minutes after injection, the animal was frozen in a dry ice/hexane mixture, supported in carboxymethylcellulose, and mounted in a Jung microtome housed in a cryostat (-20°C). Serial whole-body sections were microtomed, dried by sublimation, taped to Kodak X-ray film to generate a latent image of DFP-labeled granulocytes as they were distributed in the tissues. The radioautogram was subsequently made visible by photographic development. The following day, a sample of previously frozen guinea pig granulocytes was tagged and treated in the same manner. Radioautograms were prepared from the animal which received these cells.

RESULTS

Stability of Granulocyte-Releasing Activity

Within 60 minutes after injection, some degree of granulocytosis was observed in 24 of 29 rats which received fresh PPP (83%) and in 34 of 44 rats which received stored plasma (77%). Three hours after injection, all 73 rats demonstrated a granulocytosis, and in 68 of the animals, the granulocyte count was at least twice the pre-injection level.

The mean granulocyte increment three hours after injection of either fresh or stored plasma was greater than 6,500/mm^3 in all eight experimental groups: four groups received fresh plasma and four groups received stored plasma.

There were no significant differences in mean granulocyte increments three hours after injection in the groups of animals infused with fresh PPP and PPP stored at 4°C for 1, 7, 14 or 21 days (Table 1). The responses obtained during the three-hour period following injection of fresh PPP and PPP stored at 4°C for 1, 7 or 21 days were similar (Figure 1). There was a greater mean increment in the granulocyte count three hours after injection in animals given PPP stored at 4°C for 14 days than in the animals given fresh PPP, although this difference was not statistically significant ($p > 0.05$ by analysis of variance) (Table 1, Figure 1).

No bacterial contamination was observed in any of the plasma stored for up to 21 days.

Mobilization of Granulocytes on Successive Days

The mean increases of granulocyte counts observed in 18 animals given PPP and seven given normal plasma on two successive days are shown in Figure 2. The mean increases in granulocyte counts in the animals given PPP were essentially identical at all points on day 2 when compared with those obtained on day 1. This was true also for the animals given normal

plasma, although the increases were much smaller. The mean pre-treatment granulocyte count was slightly higher on day 2 as compared with day 1 (Table 2). In comparing the individual animals, 15 of the 18 rats given PPP demonstrated higher pre-treatment counts on day 2.

Duration of Granulocyte Increment in PPP Recipients

As seen in Figure 3, the mean maximum increment in granulocyte count of rat recipients of PPP occurred at three hours after transfusion. The mean increment seen at three hours after injection was approximately 12,000/mm^3. This increment was maintained, but not exceeded, during the subsequent three hours of observation.

Intravenous Versus Intraperitoneal Administration of PPP

Intravenous infusion of 1.0 ml of PPP/kg into eight normal rats resulted in a mean granulocyte increment of about 7,000/mm^3 three hours after injection (Figure 4). When the same dosage of PPP was given intraperitoneally, the mean granulocyte increment at three hours was slightly over 2,000/mm^3, or 29% of that seen when the same plasma was given i.v. This is equivalent to that seen previously (8,10) when normal plasma was given i.v. The intraperitoneal injection of normal saline demonstrated essentially no increment in the granulocyte count after three hours. The mean granulocyte increment in that group was approximately 300/mm^3.

Granulocyte Yields with 50% Plasma/Saline

As seen on Table 3, the mean granulocyte yield obtained from filters used to pherese eight normal rats for two hours was 6.68×10^7, with a range from 4.01 to 9.93×10^7. These eight experiments include four filters from which the granulocytes were eluted with fresh plasma and four from which the cells were eluted with frozen, previously used plasma. A study carried out before that just described and a necessary prerequisite for carrying out those experiments is summarized in Table 4. Pre-washing of the filter with saline under pressure followed by a 100% plasma wash also under pressure

resulted in the majority of the granulocytes appearing in the saline wash. By using gravity flow of the saline wash followed by 100% plasma wash under pressure, the loss of granulocytes into the saline wash was substantially reduced and the number of granulocytes in the plasma wash was increased 3-1/2-fold. However, the number of granulocytes in the plasma wash was still less than half that seen when a pre-wash was not used and 50% plasma/saline was used under pressure.

Use of Fresh and Used Plasma for Elution of Granulocytes

This study is only preliminary at this point. Table 5 compares four paired experiments. The granulocytes from one set of four nylon fiber filters were eluted with fresh plasma with the pH adjusted to 6.5 to 6.7 with ACD-A. The second set of four filters was eluted by the same plasma which had been frozen and used the following day. There was a small mean reduction of granulocyte harvest when used plasma was employed. Three of the four experiments demonstrated a reduction in granulocyte yield with a range of 13% to 22%. The fourth showed an increased yield of 18%. Additional experiments will be required to provide a definitive answer to this question.

Optimal Time for Donor Pre-treatment with PPP

To this point, this study is in a very preliminary stage. Several rats have been pheresed to provide PPP for use in pre-treating the rat donors. A base-line group of control animals (pheresed without PPP pre-treatment) has been studied. The bulk of this study still needs to be done.

Viability of Filtered Granulocytes

Because of the heavy contamination with red blood cells of the concentrate obtained without a pre-wash with saline, no size distribution of the white cells or their ability to incorporate fluorescein diacetate or to exclude ethidium bromide could be ascertained.

In the two studies in which the filters were pre-washed with saline, the granulocyte concentrate was "clean" enough to demonstrate more than 90% incorporation of fluorescein diacetate, an indication of the harvest of more than 90% viable cells. A second such experiment demonstrated greater than 95% viability of granulocytes as measured by fluorescein diacetate uptake.

Because many rat granulocytes are similar in size to the red blood cells, cell-sizing studies of rat blood may not be possible.

Radioautography to Determine the Fate of Transfused Granulocytes

As seen in Figures 5A and 5B, transfused granulocytes, whether fresh or frozen, are distributed to the lung, liver and spleen during the first 30 minutes after injection.

DISCUSSION

The results described in this report extend those carried out during the previous year. They can again be divided into two major categories; i.e., (1) those which have direct clinical significance, and (2) those which further elucidate the kinetics of granulocyte mobilization. The preponderance of clinical evidence suggests that granulocyte transfusions are effective in reducing morbidity and mortality in septic granulocytopenic patients (11-15). Our previous studies of the neutrophil-releasing activity of plasma from rat donors after filtration leukopheresis (FL) indicate that the efficiency of this method can be improved by pre-treatment with post-pheresis plasma (8). This appears to be a more physiological approach to improving the method of FL than the use of a variety of non-physiological agents now being employed for this purpose. For routine clinical use, autologous plasma containing neutrophil-releasing activity would require at least one day of storage.

The studies reported here demonstrate that rat neutrophil-releasing factor in PPP is stable for up to three weeks at 4°C. Since human NRF is apparently not stable in frozen plasma, it is suggested that human post-pheresis plasma containing NRF be stored at 4°C and liquid-stored plasma should be evaluated in clinical situations.

The ability of rats to mobilize granulocytes from tissue reserves is maintained on the second day after consecutive stimulation by neutrophil-releasing factor in post-pheresis plasma. The dose of 1.5 ml/kg given on each day is equivalent to transfusion in the human of plasma from approximately 250 ml of blood. The slight granulocytosis observed following injection of normal rat plasma is consistent with our previous observations and probably results from the heparin in the plasma, which is known to cause some degree of granulocytosis. These controls were included to determine the degree to which localized infection at the site of injection following exposure of the femoral vein on the previous day might affect the results. In the experimental group the granulocyte count prior to infusion of PPP on day 2 was slightly higher than that seen on the previous day, before injection. The rather marked increase in the granulocyte count of the control rats given

normal plasma 24 hours after exposure of the femoral vein suggests that the increase in both groups above the pre-treatment level of the previous day could be due to an infection at the operative site, rather than being a residuum of the previous day's stimulation, in spite of a normal appearance grossly of the operative site. Even so, it did not significantly affect the ability of the rats which received PPP to respond, either positively or negatively. It is not surprising that the increments in the rat granulocyte counts were similar on two successive days following administration of the dosage given. Earlier experiments showed that a dose of 1.5 ml/kg in the rat resulted in a mean increment of approximately 16,000/mm^3 (10). We had also estimated previously that if the rat granulocyte reserves, which include both the marginating granulocyte pool and the mature granulocytes in the bone marrow, are equivalent to that of humans on the basis of body mass, the number of granulocytes which could appear in the circulation following a single maximal stimulation would be of the order of 32,000/mm^3 (10). The present studies suggest that the rat granulocyte reserves are of that magnitude.

The observations that the maximum increment of the granulocyte count in the peripheral blood of rat recipients stimulated by PPP occurs at three hours demonstrate that no further advantage would lie in pre-treating granulocyte donors with PPP more than three hours before pheresis. This knowledge will allow us to confine our proposed studies of the optimal time of pre-treatment of rat donors with PPP to three hours and less.

It would have been providential if intraperitoneal injection of PPP were at all comparable to intravenous injection, especially in experiments in which the PPP will be given an hour or more before pheresis of animal recipients of the plasma. The experiments described in this report do not, however, demonstrate sufficient activity of i.p.-administered PPP to warrant its use in such experiments.

The method used to elute granulocytes from the filter when the rat model is used will depend on the purpose of the experiment. If the objective is simply to determine the efficiency of a particular method to harvest granulocytes, pre-washing of the filters with saline is not only not warranted, but

is detrimental. It adds one more variable to the technique. Removal of the red blood cells is probably also not required for transfusion studies in animals, since the infused red blood cells will not affect the ability of granulocytes to circulate. However, we observed that red cell contamination of the granulocyte concentrate will effectively prevent the use of cell-sizing techniques. The use of 50% plasma/50% saline was shown to be adequate for studies involving efficiency of filtration under various circumstances. The mean harvest of 6.68×10^7 granulocytes per filter using 50% plasma compares favorably with the 8.1×10^7 cells obtained in previous experiments (7) in which 100% plasma was used. The former figure is 82% of the latter.

Whether animal or human granulocytes are to be harvested from nylon columns, relatively substantial amounts of plasma are required to elute these cells. If it is possible to use this plasma more than once, there will be a significant advantage. Certainly, in animal experiments, the present experiments do demonstrate at least the possibility of a second use. In animal experimentation, the concern with contamination of the plasma with subsequent contamination of the granulocytes obtained when the plasma is used a second time is obviously not as great as it would be in a clinical situation. We suggest that, under the proper circumstances, and with appropriate precautions to maintain sterility and use of plasma of the correct ABO group, a second use of the plasma eluent may be indicated, for eluting human granulocytes. In addition to maintaining a closed system as nearly as possible, it may also be practical to filter out potential contaminating organisms with the use of a "Millipore" filter of appropriate pore size.

Our previous studies which demonstrated that granulocyte harvests could be significantly improved by pre-treatment of the rat donors one hour before initiation of the pheresis suggests that further improvement is possible. Experiments performed under this contract demonstrate a substantially greater increment in rat recipients of PPP at three hours after injection, as compared with one hour. In fact, there is a precipitous increase in the granulocyte count of recipients of PPP between one and three hours after injection. It is likely that the one-hour period of pre-treatment is not the optimal time. Future investigations will focus on this question. It is the central problem of all these investigations.

There are innumerable in vitro methods for determining the viability of granulocytes (16). Most of these require a degree of expertise. For definitive answers with regards to the degree of viability and the locus of specific lesions, the more sophisticated assays are generally required. However, for the purposes of the experiments being planned in collaboration with Dr. Fabian Lionetti, a simple screening method will suffice. It is our intention to carry out an in-depth study of the relative viabilities of granulocytes obtained by filtration and elutriation. Since the damage engendered by these methods is obviously quite significantly less than that resulting from freezing and thawing, such simple methods will be sufficient initially. The rather extensive experience of the Lionetti group with a combination of cytoplasmic dye exclusion and nuclear dye inclusion suggests that this method will be useful for our purposes. The preliminary studies carried out under this contract on rat granulocytes using this method has suggested that it can be used with rat granulocytes as well as with the guinea pig and baboon cells used previously. Unfortunately, it appears that the "elutriator" is not amenable for use with rat blood because of the relatively low normal concentration of granulocytes in rat blood. The comparative studies will require adapting the filtration method to the guinea pig.

It is clear from the small study of the use of radioautographic methods to determine the fate of transfused granulocytes in the body that this method can provide significant information in this regard. The concentration of radioactivity in the lungs, liver and spleen of both the fresh and frozen granulocytes suggests that the radioactivity which was incorporated into the granulocytes remained incorporated for 30 minutes rather than being eluted and distributed through the body. This is a primary requisite for successful methodology using radioautographic techniques. Although the study presented here is very preliminary and of a very limited nature, we believe that it demonstrates an important potential for obtaining information on the effects of a variety of granulocyte preparations.

Because of the cost and complexity of such studies, further investigations of this nature cannot be undertaken under this contract.

CONCLUSIONS

Plasma obtained from rats which have been leukopheresed can be used to increase the granulocyte yields with leukopheresis procedures in subsequent donors. This results from the activity of humoral factors elaborated by the animal during the pheresis. The neutrophil-releasing activity of post-pheresis plasma is stable for up to three weeks at 4°C. When aliquots of the identical PPP are used in the same animal on successive days, the increased pre-treatment count observed on the second day is probably the result of a sub-clinical infection at the injection site rather than of a residuum of the previous day's stimulation. The maximum granulocyte increment observed following injection of PPP occurs at three hours and is maintained for at least the following three hours. Intraperitoneal injection of PPP is ineffective in stimulating the release of neutrophils from body reserves for up to three hours after injection. The use of 50% rather than 100% plasma for elution of granulocytes from nylon filter columns results in granulocyte yields which are approximately 20% less. However, an 80% yield may be perfectly adequate for most of the proposed studies. This technique will result in the saving of substantial volumes of plasma. It is possible to use the plasma/saline eluent at least twice in the animal model being used without substantial loss of efficiency. We have shown that the dye inclusion/exclusion method of Dankberg and Persidsky for determining granulocyte viability can be adapted to the study of rat granulocytes but that the "elutriator" is probably not an effective instrument for separating granulocytes from rat blood. Preliminary non-optimal studies have suggested that whole-body radioautographic methods are useful in determining the viability and fate of a variety of preparations of transfused granulocytes.

SIGNIFICANT ACCOMPLISHMENTS

We have extended the studies of the kinetics of granulocyte mobilization following injection of post-leukopheresis plasma which were begun in year one of this contract. We have recommended the liquid (4°C) storage of PPP for use in clinical studies. We have recommended that in clinical studies pre-treatment of granulocyte donors should be carried out as long as possible (but not more than three hours) before leukopheresis. This results from the observations that the mean granulocyte increment occurs at three hours after injection of PPP. We have re-initiated studies of granulocyte harvest in rats under a variety of conditions and have shown that dye inclusion/exclusion methods and whole-body radioautography can be used effectively to study basic and applied physiology of harvested and transfused rat or guinea pig granulocytes. Preliminary studies have demonstrated that certain proposed studies will require the development of a guinea pig model for filtration leukopheresis.

The following papers have been published or accepted for publication:

1. Roy, A., and Ramirez, M. The effect of plasma from leukopheresed rats in normal recipients: a dose-response study. Accepted by "Experimental Hematology."

2. Roy, A. Methods for assaying viability of frozen-thawed granulocytes. Accepted by "Cryobiology."

3. Roy, A., Ramirez, M., and Valeri, C.R. Stability of neutrophil-releasing activity of plasma obtained from leukopheresed rats and stored at 4°C. Accepted by "Transfusion."

4. Roy, A., and Ramirez, M. Mobilization of granulocytes on successive days with plasma from leukopheresed rats. Published in: Blood Leukocytes: Function and Use in Therapy (C. Högman, K. Lindahl-Kiessling and H. Wigzell, eds.), Reklam & Kalalogtryck, Uppsala, Sweden, 1977.

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TABLE 1

MEAN GRANULOCYTE INCREMENTS THREE HOURS AFTER INJECTION

OF FRESH OR STORED POST-PHERESIS PLASMA INTO NORMAL RATS

Days of Storage	Experiments	Mean Increment of Granulocyte Counts (MIGC) /mm^3 ± S.D.*	MIGC (Stored) / MIGC (Fresh) (%)
0	7	6,706 ± 2,059	
1	10	6,757 ± 4,182	101
0	6	7,382 ± 2,883	
7	10	6,501 ± 3,219	88
0	6	7,124 ± 3,518	
14	11	11,855 ± 5,074	167
0	10	9,700 ± 7,505	
21	12	9,988 ± 7,193	103

* standard deviation

TABLE 2

CHANGE IN GRANULOCYTE COUNTS AFTER INJECTION OF PLASMA

FROM LEUKOPHERESED RATS INTO NORMAL RECIPIENTS

ON TWO SUCCESSIVE DAYS

PPP-Treated

	Mean Initial Gran. Count/mm^3	Mean Gran. Count 3 Hours after Tx	Mean Gran. Increase 3 Hours after Tx
Day 1	4,137 ± 2,648*	20,171 ± 5,716	16,034 ± 6,534
Day 2	5,972 ± 3,535	21,647 ± 6,171	15,675 ± 4,927

Normal Plasma

Day 1	3,350 ± 504	6,340 ± 3,032	2,990 ± 2,699
Day 2	8,975 ± 3,055	12,623 ± 5,098	3,648 ± 3,125

* mean ± standard deviation

TABLE 3

GRANULOCYTE YIELD FROM FILTERS AFTER LEUKOPHERESIS OF NORMAL RATS

Experiment No.	Yield of Granulocytes x 10^7
1	8.06
2	9.93
3	4.01
4	6.50
5	6.27
6	8.30
7	4.75
8	5.64
Mean	6.68

TABLE 4

HARVEST OF GRANULOCYTES BY VARIOUS METHODS

	Force of Saline Wash	Yield of Granulocytes: Saline Wash ($\times 10^7$)	Yield of Granulocytes: Plasma/Saline Wash ($\times 10^7$)	Yield of Granulocytes: Plasma Wash ($\times 10^7$)	Yield of Granulocytes: Total Gran. ($\times 10^7$)
50% plasma/ saline	under pressure	-	7.34	-	7.34
100% plasma	under pressure	5.50	-	1.07	6.57
100% plasma	gravity flow	3.09	-	3.63	6.72

TABLE 5

EFFECT OF FRESH OR PREVIOUSLY USED PLASMA/SALINE

IN HARVESTING GRANULOCYTES FROM NYLON FILTERS

	Granulocyte Yield x 10^7		Difference in Yield x 10^7	
	First Day	Second Day	Day 1 - Day 2	% Difference
	8.06	6.27	- 1.79	- 22
	9.93	8.30	- 1.63	- 16
	4.01	4.75	+ 0.74	+ 18
	6.50	5.64	- 0.86	- 13
Mean	7.13	6.24		

FIGURE 1

MEAN INCREASE IN GRANULOCYTE COUNT OF RATS GIVEN FRESH (—) OR STORED (--) POST-PHERESIS PLASMA

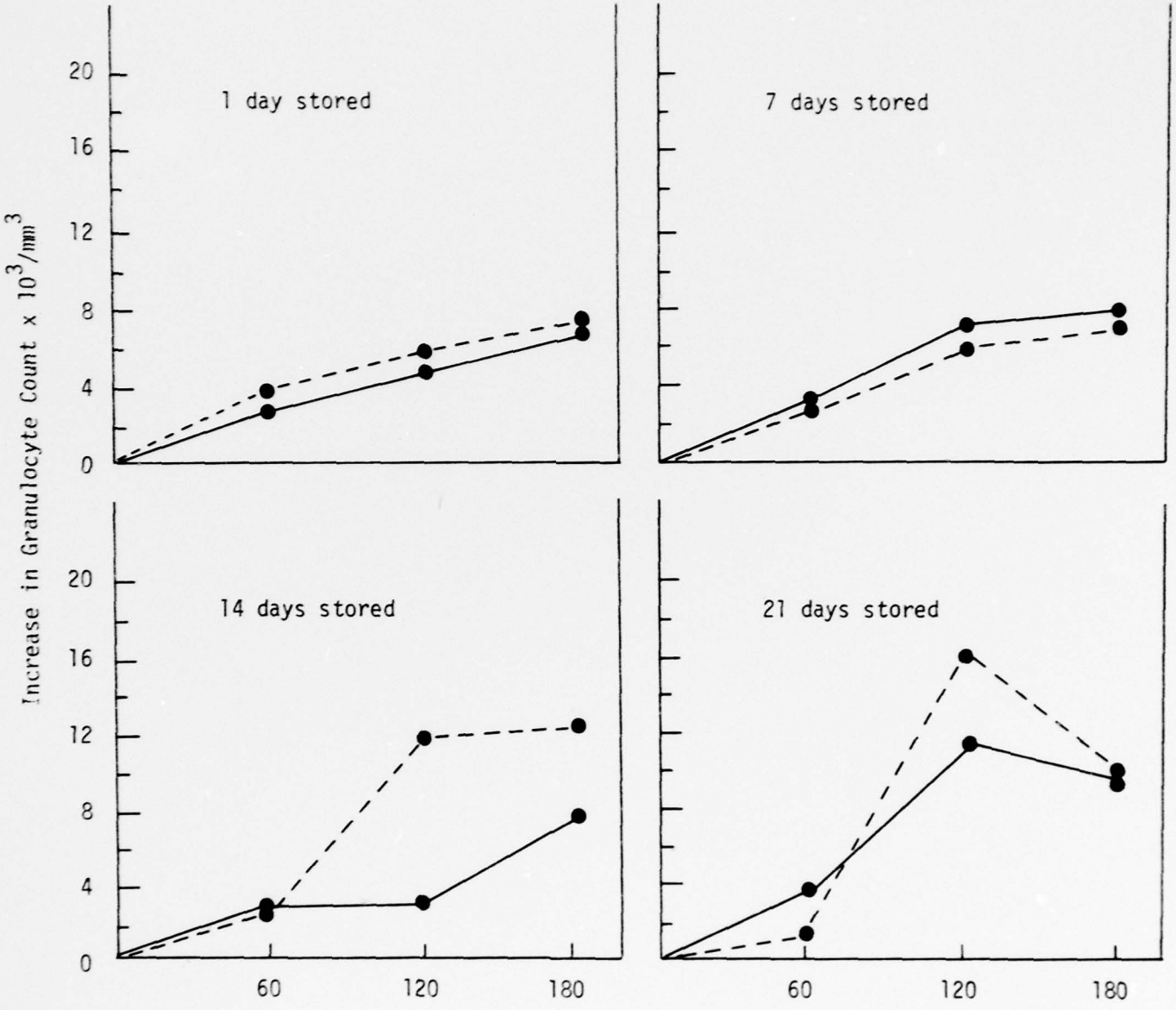


FIGURE 2

INCREASE IN GRANULOCYTE COUNT OF RATS GIVEN POST-PHERESIS OR NORMAL PLASMA ON SUCCESSIVE DAYS

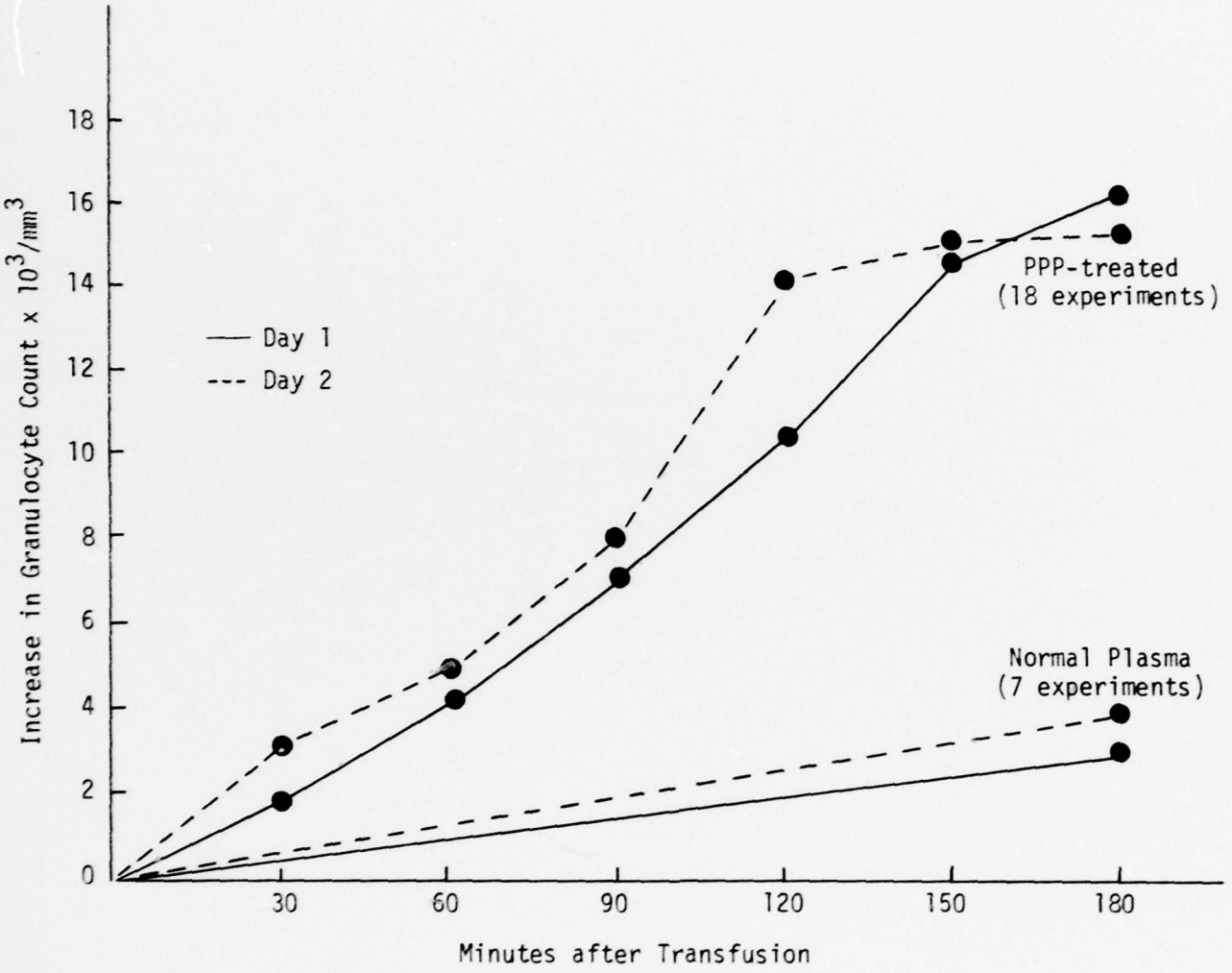


FIGURE 3

MEAN GRANULOCYTE INCREASE FOLLOWING TRANSFUSION OF PLASMA FROM LEUKOPHERESED RATS INTO NORMAL RECIPIENTS

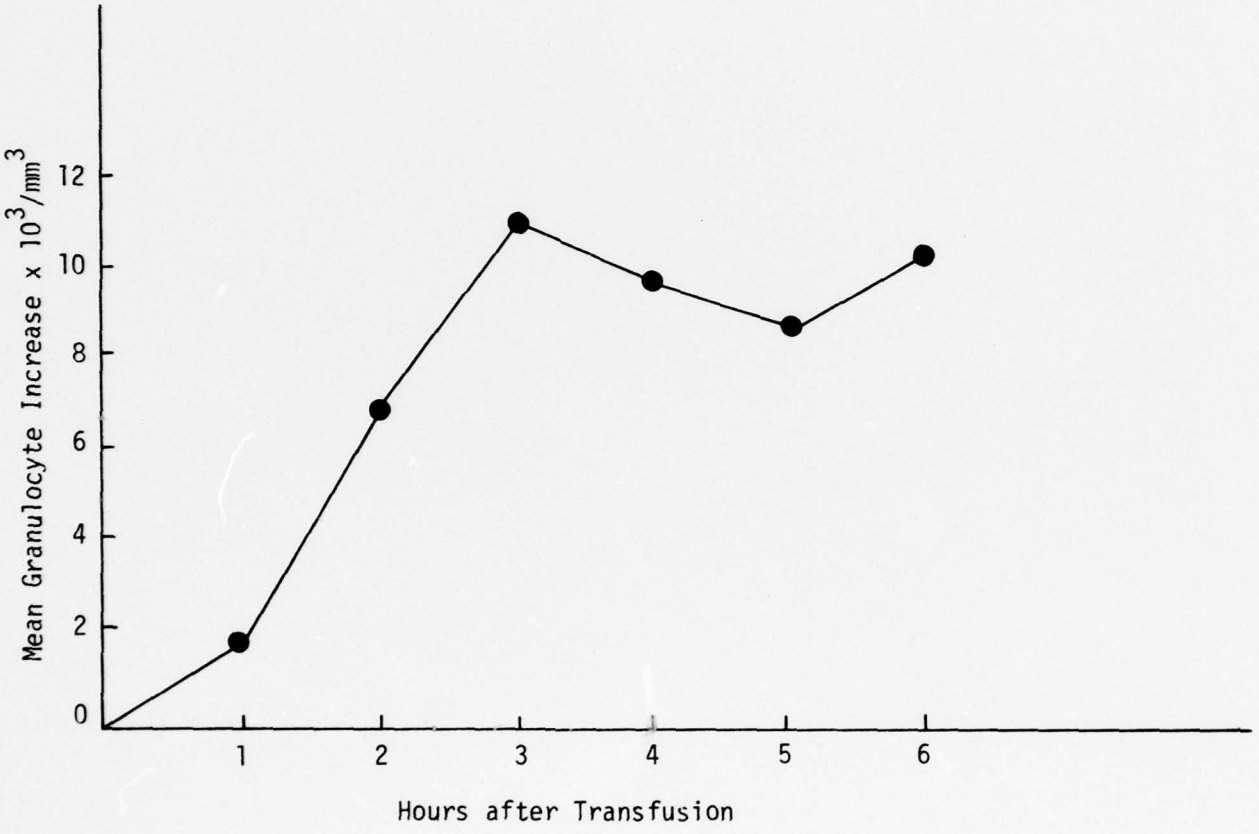
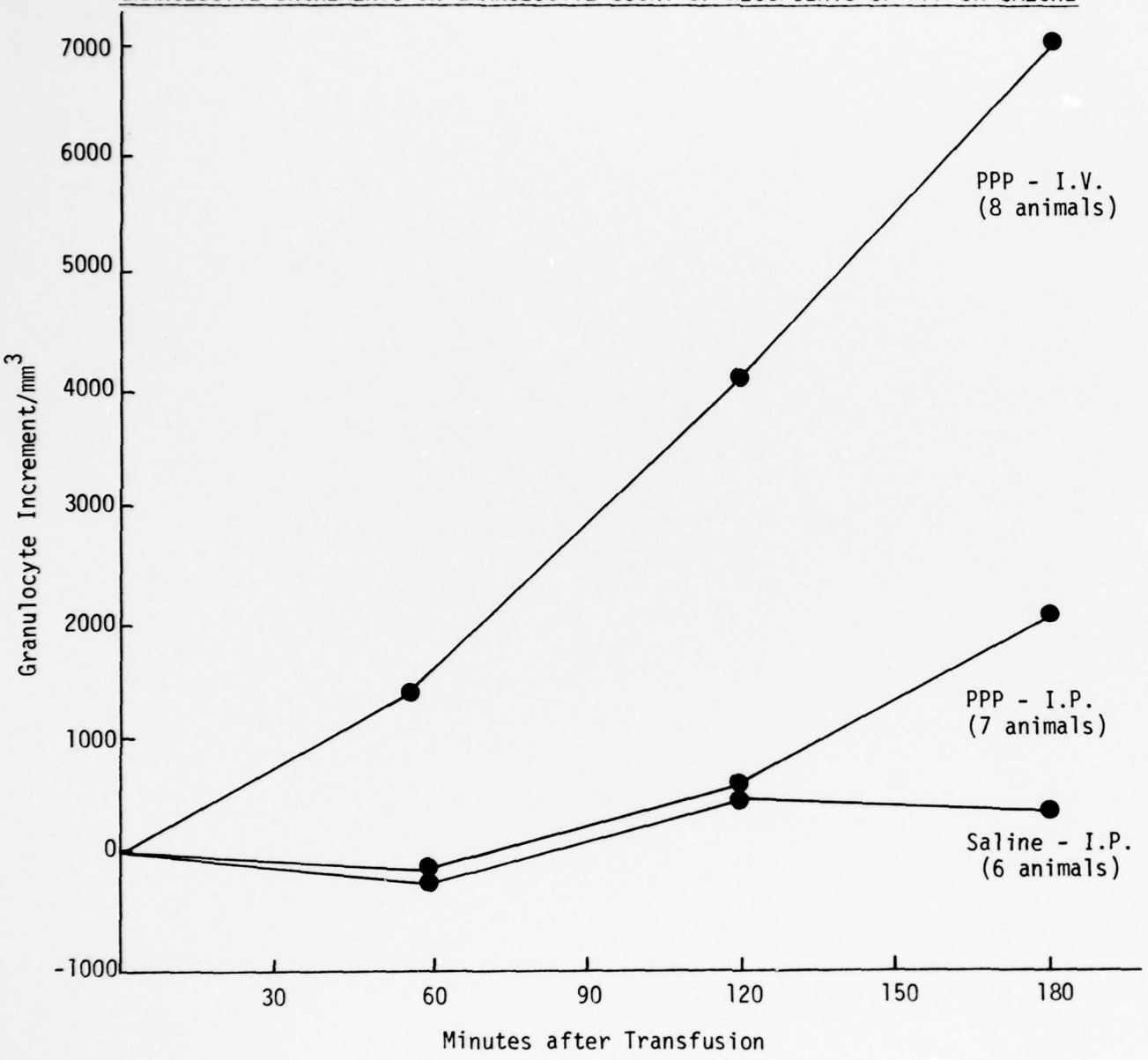


FIGURE 4

GRANULOCYTE INCREMENTS IN GRANULOCYTE COUNT OF RECIPIENTS OF PPP OR SALINE



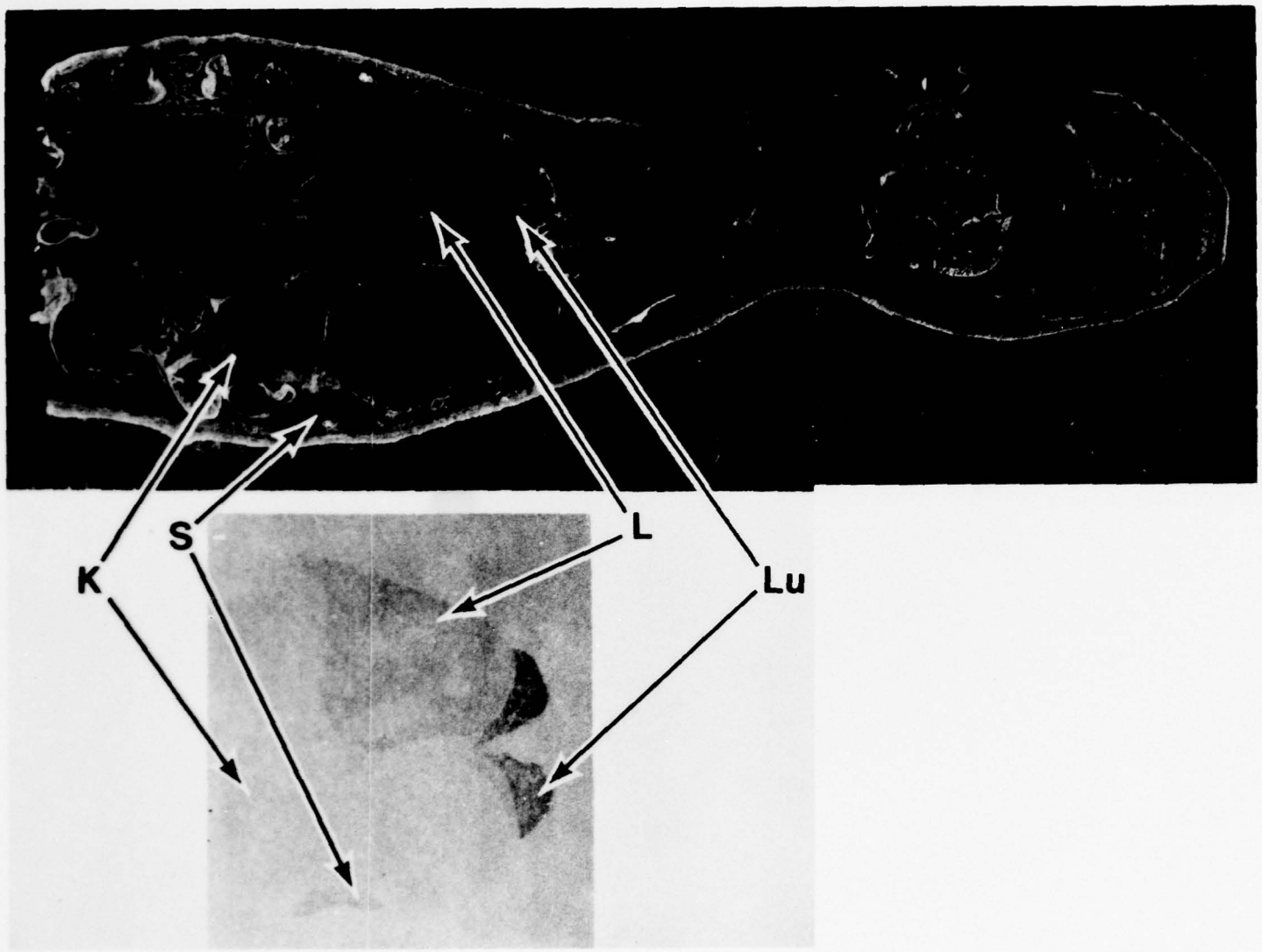


FIGURE 5A. Whole-body section (above) and corresponding whole-body radioautogram (below) of guinea pig transfused with frozen-thawed guinea pig granulocytes tagged with radiolabeled DFP. Labeled granulocytes are seen in the kidney (K), spleen (S), liver (L), and lungs (Lu).

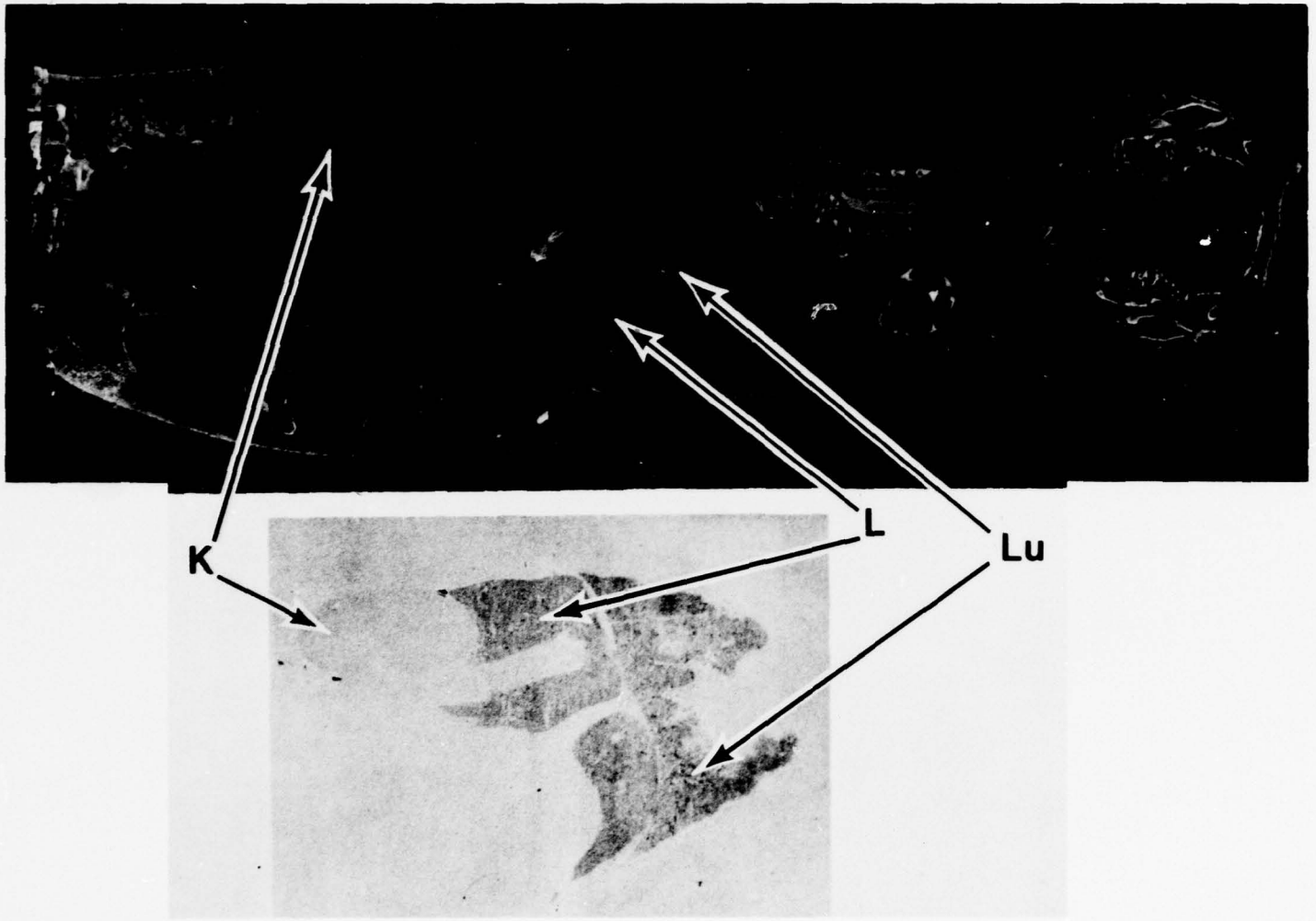


FIGURE 5B. Whole-body section (above) and corresponding whole-body radioautogram (below) of guinea pig transfused with fresh guinea pig granulocytes tagged with radiolabeled DFP. Labeled granulocytes are seen in the kidney (K), liver (L), and lungs (Lu). No statement can be made regarding the spleen, since the spleen does not appear in this particular plane of section.

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Plasma obtained from rats which have been leukopheresed can be used to increase the granulocyte yields with leukopheresis procedures in subsequent donors. This results from the activity of humoral factors elaborated by the animal during the pheresis. The neutrophil-releasing activity of post-pheresis plasma is stable for up to three weeks at 4°C. When aliquots of the identical PPP are used in the same animal on successive days, the increased pre-treatment count observed on the second day is probably the

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20. result of a sub-clinical infection at the injection site rather than of a residuum of the previous day's stimulation. The maximum granulocyte increment observed following injection of PPP occurs at three hours and is maintained for at least the following three hours. Intraperitoneal injection of PPP is ineffective in stimulating the release of neutrophils from body reserves for up to three hours after injection. The use of 50% rather than 100% plasma for elution of granulocytes from nylon filter columns results in granulocyte yields which are approximately 20% less. However, an 80% yield may be perfectly adequate for most of the proposed studies. This technique will result in the saving of substantial volumes of plasma. It is possible to use the plasma/saline eluent at least twice in the animal model being used without substantial loss of efficiency. We have shown that the dye inclusion/exclusion method of Dankberg and Persidsky for determining granulocyte viability can be adapted to the study of rat granulocytes but that the "elutriator" is probably not an effective instrument for separating granulocytes from rat blood. Preliminary non-optimal studies have suggested that whole-body radioautographic methods are useful in determining the viability and fate of a variety of preparations of transfused granulocytes.

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