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KINETICS OF NEUTROPHIL-RELEASING ACTIVITY OF POST-LEUKOPHERESIS--ETC(U)
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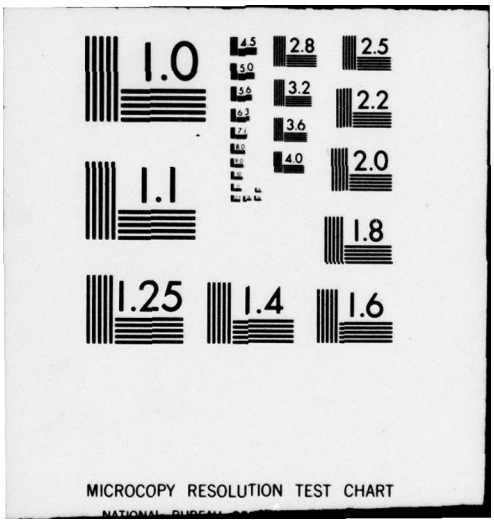
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OFFICE OF NAVAL RESEARCH

Contract N00014-76-C-0489

Task No. NR207-062

ANNUAL REPORT NO. 3

KINETICS OF NEUTROPHIL-RELEASING ACTIVITY OF POST-LEUKOPHERESIS PLASMA

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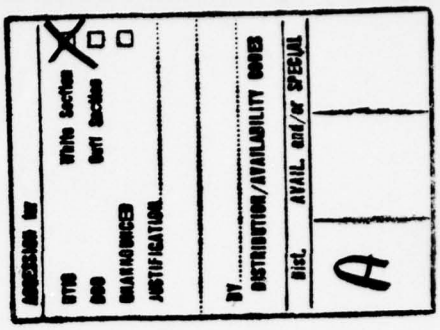

INTRODUCTION

There are two primary objectives of the studies being carried out under this contract:

1. to determine the clinical relevance of earlier observations that plasma obtained from animals which have undergone filtration leukopheresis given to normal animals results in an increase in the recipients' granulocyte counts; and

2. to use the phenomenon of the induction of granulocytosis by this means to elucidate normal granulocyte kinetics.

We have shown in earlier experiments that the granulocytopoietic properties of plasma obtained from granulopheresed animals almost certainly resides in a humoral factor which is elaborated by the body in response to removal of large numbers of granulocytes which we (1) and others (2) have called "neutrophil releasing factors" (NRF). We have demonstrated that the granulocytopoietic activity of NRF is dose related, that the magnitude of its elaboration depends essentially on the duration of pheresis and, therefore, on the number of granulocytes removed from the body (3). We have taken advantage of this humoral factor to improve the yield of granulocytes harvested by filtration leukopheresis by pre-treating potential animal granulocyte donors with post-pheresis plasma (PPP). Studies carried out during earlier phases of the present contract have shown that the NRF activity in post-pheresis plasma is stable during liquid storage at $4^{o}C$ (4) and during frozen storage at $-20^{o}C$ (5) for at least two weeks.

The primary goal of these studies also involved practical considerations of the characteristics of timing and dosage of PPP preliminary to clinical studies of its usefulness to improve the technique of harvesting granulocytes for use in septic patients. However, a very important by-product of these investigations relates to basic mechanisms of granulopoiesis. We have, in the course of these studies, obtained important information regarding the kinetics of granulocyte mobilization from the marginating granulocyte pool and the bone marrow (3,4,6,7,8).

During the period of the present task (1 February 1978 to 31 January 1979), these studies have been extended to include:

1. further studies to determine the optimal time to pretreat rat granulocyte donors with PPP;

2. the effect of injection of PPP in recipients during the first 30 minutes after treatment;

3. determination of a correlation between the donor granulocyte count at the termination of granulopheresis and the granulocyte yield;

4. demonstration of a direct correlation between the initial (prepheresis) donor granulocyte count and the granulocyte increment observed in the donor following a standard period of pheresis;

5. effect of age (weight) on the granulocyte increment during granulopheresis;

6. further studies on a second use of 50% plasma/50% saline for elution of granulocytes from nylon filters;

7. studies on the use of eluents other than homologous plasma or plasma/saline for harvesting granulocytes from nylon filters which include:

   (a) hydroxyethyl starch,
   (b) phosphate buffered saline plus human albumin, and
   (c) phosphate buffered saline plus bovine albumin.

8. modification of the method for staining blood smears to accommodate the staining of smears obtained from filter eluates of granulocytes;

9. additional studies of whole-body radioautographs of guinea pigs infused with fresh or frozen homologous granulocytes (in collaboration with Dr. Fabian Lionetti, Dr. Robert Liss and Dr. C. Robert Valeri);

10. modification of the rat filtration leukopheresis method for granulopheresis of guinea pigs;

11. studies of kinetics of granulocyte increments obtained in rats under the following conditions:

    (a) injection of PPP followed by pheresis,
    (b) PPP alone, and
    (c) pheresis alone.

METHODOLOGY

Optimal time to pre-treat rat granulocyte donors with post-pheresis plasma

We showed previously (1) that the yield of granulocytes from leukopheresed rats could be improved by pre-treatment of the donors with plasma from leukopheresed rats (PPP). Only the rats pre-treated one hour before infusion were studied in these earlier experiments. At that time a dosage of PPP from 25 to 100% greater than the presently used standard dose of 1.5 ml/kg was used. Studies previously carried out under this contract subsequent to those early studies, demonstrated that the granulocyte counts continue to increase for three hours after transfusion of the PPP. Since the improved yield of granulocytes from donors pre-treated in that manner results presumably from the greater availability of granulocytes mobilized by the neutrophil releasing activity of the plasma and since such availability becomes greater with time after injection of the PPP, investigations were initiated to demonstrate the optimal time after injection of PPP to initiate pheresis such that an optimal harvest of granulocytes would occur.

Sprague-Dawley rats weighing 350 to 450 grams were leukopheresed as described previously (9). Following pheresis, the rats were exsanguinated and the plasma was separated by centrifugation of the whole blood. The plasma was frozen at -20^{o}C for future use. Additional groups of rats were treated as follows:

1. eight rats were pheresed for two hours;
2. nine rats were pre-treated with 1.5 ml/kg of PPP given intravenously one hour before initiation of pheresis;
3. four rats were pre-treated with 1.5 ml/kg of PPP given intravenously two hours before initiation of pheresis.

Before any treatments (injections of PPP or pheresis) were carried out, cannulae were inserted into a jugular vein and carotid artery and the animals were anticoagulated with Heparin-sodium, 400 I.U. per animal. In animals to be pre-treated with PPP, the material was injected via the jugular vein cannula. An arterio-venous shunt was then constructed by connection of the arterial and venous cannulae. At the appropriate time after injection of PPP, a nylon-wool packed filter was inserted into the system in the standard manner.

In all groups, following two hours of pheresis, the filters were removed from the system and the granulocytes were eluted with 35 ml of phosphate buffered saline enriched with bovine albumin (prepared as described later in this section). The yield of granulocytes was calculated following determinations of the total white blood cell count, the differential WBC count and volume of the eluate.

Increments in granulocyte counts during the first 30 minutes after injections of post-pheresis plasma

This study was done in response to a report that PPP given to human granulocyte donors 15 to 20 minutes before initiation of granulopheresis did not result in a significant increase in the granulocyte harvest (10). It was our impression, although we had no data to support this, that the increment in granulocyte count during the first 30 to 60 minutes after PPP was inconsequential.

In order to determine the degree of granulocyte mobilization in the rat within the time frame covered by the reported clinical experiments, post-pheresis plasma was obtained in the usual manner. The standard dose of 1.5 ml/kg was injected into the exposed femoral vein of 19 normal Sprague-Dawley rats weighing 120 to 240 grams.

The studies were carried beyond the 30 minute period to one hour in order to substantiate that these animals were comparable to those done over the previous 18 months. Granulocyte counts were determined before and 10, 20, 30, 40, 50 and 60 minutes after transfusion of the PPP. The increment in granulocyte count was calculated for each time period after infusion of PPP.

Correlations between donor pre-pheresis granulocyte count and granulocyte harvest

During the course of the present studies, the eluent for washing granulocytes from nylon filters following two hours of granulopheresis was changed. The earlier studies employed 50% normal rat plasma in normal saline. Later studies made use of phosphate buffered saline enriched with bovine albumin (PBS + albumin), as described later in this section. In order not to confuse the interpretation of the experiments described here, the experiments in which the two eluents were used are plotted separately.

Our contention since the initiation of these studies has been that the harvest of granulocytes ought to bear a direct relationship to the granulocyte count of the animal. We chose to consider the granulocyte count in the animal at the end of pheresis to determine the correlation with granulocyte harvest. The data from each of the ten studies using plasma/saline as the eluent were derived from a single study and each was treated in the same way, i.e., the animals were pheresed for two hours; a granulocyte count was obtained and the granulocytes were eluted from the filter with 35 ml of 50% plasma/50% saline. The total number of granulocytes in the eluent was calculated.

For the second group of experiments in which PBS - albumin was used as the eluent, each of 32 experiments were included in the calculations regardless of what had been done to the animal to affect

its granulocyte count prior to elution. Our consideration here was only to correlate the post-pheresis count with the granulocyte yield. Therefore, these experiments include animals which were pheresed for two hours, as well as those given PPP one or two hours before pheresis. As in the previous group, immediately after granulopheresis, the filters were washed with 35 ml of eluent, and the total number of eluted granulocytes was calculated. A determination was made of the correlation between the post-pheresis granulocyte count of the donor and the granulocyte yield.

Correlation between the donor pre-pheresis granulocyte count and the post-pheresis granulocyte increment

An analysis of 58 comparable experiments was carried out in which the increment in the granulocyte count of animals pheresed for two hours was correlated with the pre-pheresis granulocyte count. A WBC and differential WBC count were obtained before and immediately after granulopheresis. The experiments were divided into three groups:

1. pre-pheresis counts less than 2,000/mm^3;
2. pre-pheresis counts between 2,000 and 3,999/mm^3;
3. pre-pheresis counts of 4,000/mm^3 or greater.

The mean increment of granulocyte counts for each of the three groups was calculated.

Effect of weight on the granulocyte increment during granulopheresis

The weight of rats is roughly proportional to their age. The weanling weighs 80 to 100 grams. Routine cannulation of the jugular vein and carotid artery preparatory to pheresis can be done in animals weighing 150 grams or more. Larger rats (>300 grams) tolerate the procedure significantly better than do smaller ones and the yield of

plasma is also much greater. Since both of these considerations are important, and the larger the rat the more convenient are the procedures which can be carried out. However, mechanical considerations must be subordinated to the integrity of the experiment as well as to the need to obtain optimal results. With these considerations in mind an analysis of 62 experiments was carried out in which the animals were divided into four groups according to weight:

1. less than 300 grams;
2. between 300 and 399 grams;
3. between 400 and 499 grams; and
4. 500 grams or more.

Granulocyte increments had been determined for each of the animals. All animals had undergone the standard two hour granulopheresis. The mean granulocyte increment was calculated for each group. An analysis was made to determine the relationship between these two factors.

Further studies on the second use of plasma/saline for elution of granulocytes

We had reported previously (11) that 50% plasma/50% saline could be substituted for 100% plasma in eluting granulocytes from nylon filters following filtration leukopheresis of normal rats. We also demonstrated that this eluent could be used more than once. This study was carried out in an attempt to reduce the cost of granulocyte elution. Since the last reporting period, we have added an additional pair of such experiments, and the results of these experiments have been included in this report and compared with those reported previously.

Studies on the use of non-plasma containing media for eluting granulocytes from nylon filters

Plasma containing media have traditionally been used in clinical studies of granulocyte yield using the method of filtration leukopheresis. Obtaining large volumes of plasma for this purpose is generally not a problem in blood banks using this technique. However, in small animal studies, plasma for elution of granulocytes must be obtained by exsanguination of donor animals. Even with the use of 50% plasma/50% saline, rather than 100% plasma (which has been shown to be most efficacious for this purpose), at least two rats must be sacrificed to provide sufficient plasma for elution of a single filter. Several alternatives to the use of homologous plasma have been investigated as possible replacements for plasma containing eluents:

(a) Hespander [Japanese hydroxyethyl starch (HES)],
(b) phosphate buffered saline plus human albumin, and
(c) phosphate buffered saline plus bovine albumin.

The HES was used as a 100% solution or diluted 1:1 with saline. The phosphate buffered saline plus albumin was prepared as follows: (1) stock solutions of 0.2M monobasic sodium phosphate and 0.2M dibasic sodium phosphate were made; (2) the phosphate buffer was prepared by adding 6.25 ml of the monobasic sodium phosphate to 3.75 ml of the dibasic sodium phosphate; and (3) the elution medium consisted of 14.0 ml of the phosphate buffer, 7.0 ml of ACD anticoagulant, 31.5 ml of isotonic-saline acid, and 17.5 ml of 5% human or bovine serum albumin. The pH was adjusted to between 6.2 and 6.5 by addition of an appropriate volume of disodium phosphate.

Normal Sprague-Dawley rats were pheresed for two hours by the standard method. Following pheresis, the filters were eluted with 35 ml of one of the four test eluents. As routinely recommended and practiced, the filters were constantly tapped during elution and the eluent was pushed through the filter under gentle pressure at a rate of approximately 7 ml per minute. Granulocyte counts were obtained in triplicate; WBC smears were obtained in duplicate; and the volume of the eluent was measured. The total number of granulocytes harvested from the filters were calculated using the results of these measurements. The results were compared with those obtained using the standard plasma-containing eluents.

Modification of methods for staining smears from the eluates

The absence of a large concentration of protein, as represented by the plasma used in previous experiments, apparently altered the staining characteristics of granulocytes eluted in the presence of low concentrations of protein in the non-plasma containing eluents. Smears of whole blood or eluates obtained containing a high concentration of plasma can be readily and clearly stained by the rapid method using "Quik-stain" (modified Wright's stain) for the purpose of obtaining differential white blood cell counts. This normally includes immersion of the slide for 10 seconds in the stain, and 20 seconds in a distilled water bath. Using the same staining regimen, the white blood cells on smears of eluates from nylon filters washed with non-plasma containing medium are small and very dark. It is not possible to differentiate among the white blood cells. A study was carried out to compare conventional Wright's stain and Quik-stain in various times and combinations to optimize the reading of the differential WBC smears.

Whole-body radioautography of guinea pigs infused with fresh or frozen-thawed granulocytes.

In cooperation with Drs. Robert Liss, Fabian Lionetti, and C. Robert Valeri, the studies initiated during the previous reporting period with regard to the whole-body disposition of infused fresh or frozen-thawed granulocytes by radioautography were amplified. We had reported previously (11) the results of a quick and non-optimal study using guinea pig granulocytes obtained with the use of the "Elutriator." The granulocytes were tagged with ^{14}C-DFP, concentrated and injected intravenously into normal guinea pigs via the exposed femoral vein. Thirty minutes after injection, the animals were frozen in a dry ice/hexane mixture supported in carboxymethyl cellulose and mounted in a Jung microtome housed in a cryostat (-20^{o}C). Serial whole-body sections were microtomed, dried by sublimation, and taped to Kodak x-ray film to generate a latent image of DFP-labeled granulocytes as they were distributed in the tissues. The radioautograms were subsequently made visible by photographic development. The previous report documented the results of studies in one animal which had received fresh granulocytes and one which had received frozen-thawed cells.

During this reporting period, we have added two additional pairs of animals given granulocytes and an additional (control) animal given only ^{14}C-DFP. The control animal was included to validate the experiments in which cells were infused.

In addition to preparation of the radioautogram, densitometry was carried out on appropriate organs including blood, kidney, spleen, lung and liver.

Filtration leukopheresis in the guinea pig

Future studies in collaboration with Dr. Fabian Lionetti are projected. These studies project separation of granulocytes from whole blood of small animals using techniques of counterflow elutriation. Preliminary collaborative studies with Dr. Lionetti of elutriation of rat blood have shown that harvesting of granulocytes by this method is difficult, if not impossible, because of the relatively small percentage of granulocytes in rat blood (approximately 18%). With the potential for the future collaborative studies in mind it would be useful to determine whether the guinea pig is amenable to the use of filtration leukopheresis. It could not be assumed that this would be possible because of the nature of the anatomy of the guinea pig. With its short, thick neck, it was not certain whether the required cannulation procedures attendant to the technique of filtration leukopheresis would be possible. Consequently, an investigation was carried out to determine the suitability of the guinea pig for studies using filtration leukopheresis.

Kinetics of granulocyte increments

An analysis was made to determine the maximum granulocyte increments which are possible following a variety of treatments, i.e.:

1. two hours of granulopheresis alone;
2. one hour of PPP alone;
3. two hours of PPP alone;
4. three hours of PPP alone;
5. four hours of PPP alone;
6. one hour of PPP plus two hours of pheresis; and
7. two hours of PPP plus two hours of pheresis.

The objective of this analysis was to determine the maximum effect which could be expected under the greatest impetus for mobilizing granulocytes by postpheresis plasma, filtration leukopheresis, and a combination of these two modalities. We hoped to obtain additional information on the kinetics of granulocyte mobilization and granulopoiesis.

In the first group of experiments, granulocyte counts were obtained before and after two hours of pheresis.

In the second through fifth groups, granulocyte counts were obtained one, two, three, or four hours, respectively, after injection of 1.5 ml/kg of PPP in the usual manner.

In the sixth and seventh groups, 1.5 ml/kg of PPP were injected i.v., one or two hours, respectively, before carrying out two hours of pheresis. Granulocyte counts were obtained before injection of PPP just before initiation of pheresis and immediately following pheresis.

Granulocyte increments were calculated at the appropriate times during each of the experiments.

RESULTS

Optimal time of pretreatment with post-pheresis plasma

Eight rats pheresed for two hours without pretreatment with post-pheresis plasma yielded a mean granulocyte increment of 9.69 x 10^7 granulocytes (Table 1). When nine rats were given 1.5 ml PPP/kg of body weight, one hour before pheresis the mean yield of granulocytes was 16.34 x 10^7, an increase over the non-treated controls of almost 70%. Pre-treatment with PPP two hours before a two hour pheresis resulted in a mean yield of 15.53 x 10^7 granulocytes. However, there were only four experiments in that group to this point. Additional studies will be needed to validate this level of granulocyte harvest. Studies will also be carried out in animals pre-treated three hours before pheresis.

An additional six animals were used as controls. The flow rate of blood through the filters in these animals was below that considered as acceptable for pheresis. The mean granulocyte yield in these animals following two hours of unsatisfactory pheresis was 0.51 x 10^7 granulocytes.

Granulocyte increments during the first hour after PPP injection

As seen in Figure 1, there was a small but steady increase in the mean granulocyte count of 19 normal rats which received 1.5 ml/kg of post-pheresis plasma. An increase above the preceding time period was seen at 10, 20, 30, 40, 50, and 60 minutes after transfusion. One hour following injection of PPP, the granulocyte count averaged approximately 3500/mm^3 above the pre-treatment count.

Correlation between donor post-pheresis granulocyte count and granulocyte harvest

As seen in Figure 2, there was a rough direct correlation between the granulocyte count following filtration leukopheresis and the granulocyte harvest among 10 experiments in which 50% plasma/50% saline was used as the eluent. Four of six (67%) animals with post-pheresis counts below 15,000/mm^3 demonstrated granulocyte yields below 7 x 10^7 cells while only one of four (25%) with post-pheresis granulocyte counts above 15,000/mm^3 yielded harvests below 7 x 10^7 cells. There were too few experiments in this study to carry out an analysis to determine statistical significance of these data.

In the second group of 32 experiments in which phosphate buffered saline plus bovine albumin was used to elute the granulocytes from the filters, a similar analysis was carried out. As shown in Figure 3 and Table 2, there was again a direct correlation between the post-pheresis granulocyte count and the granulocyte yield. An analysis carried out strictly according to that done previously demonstrated that when the post-pheresis count was below 15,000/mm^3, 8 of 15 (53%) of the yields were below 7 x 10^7 granulocytes while with post-pheresis counts above 15,000, 14 of 18 (78%) yields were above 7 x 10^7.

If the yields in excess of 13 x 10^7 are analyzed, only 1 of 15 (7%) of the animals with post-pheresis granulocyte counts below 15,000 reached that level while 11 of 18 (61%) with post-pheresis granulocyte counts above 15,000 yielded 13 x 10^7 or more cells. Both these correlations are significant ($<.05$ and $<.005$, respectively) by the chi square method of analysis.

Pre-pheresis granulocyte count vs. post-pheresis increment

As seen in Table 3, the mean increment in the granulocyte count in 12 normal rats by the end of two hours of filtration leukopheresis was lowest (9,189/mm^3) in animals with pre-pheresis granulocyte counts less than 2,000/mm3. There was a markedly greater increment in rats with initial counts between 2,000 and 3,999 and in those with initial counts greater than 3,999 [14,556 (+58%) and 14,808 (+61%)]. However, there was no difference in the increments seen in the latter two groups.

Donor Weight (Age ?) vs. the granulocyte increment during pheresis

Table 4 demonstrates a greater mean increment of granulocyte counts in donor animals undergoing two hours of filtration granulopheresis in smaller (younger) rats than in larger (older) ones. In the group weighing less than 300 grams, the granulocyte increment averaged less than 5,000/mm^3.

In the animals weighing 300 to 399 grams, a doubling of the increment over that seen in the smaller group was observed. The greatest increment (mean - 17,413) was seen in the group weighing between 400 and 499 grams. This represents an increment more than 3 1/2 times that seen in the group of small animals. There was no greater increment observed in the heaviest (oldest) rats.

Second use of 50% plasma/50% eluent

We had shown previously (8) in four paired experiments that plasma/saline eluent could be used more than once without loss of efficiency in harvesting granulocytes. A fifth paired experiment has been added to the original four. This experiment was consistent with those done previously. Table 5 demonstrates the

results of the original four experiments together with the one added during this reporting period. We will add additional experiments as time permits.

Non-plasma containing elution media

1. Hydroxyethyl starch (HES)

Only half the experiments done using 50% HES/50% saline or 100% HES were evaluable because, in many of the experiments, all the white blood cells eluted from the filters appeared "smudged" and could not be identified. Without adjustment of the pH, HES or HES/saline was under 6.0. Adjustment of the pH to 6.30 with ACD was helpful in reducing damage to the cells but some damage was still apparent. There were too few good experiments to evaluate. Further work on this material as an eluent of granulocytes from nylon filters will be carried out during the next reporting period.

2. Phosphate buffered saline plus human albumin

The use of phosphate buffered saline enriched with 2% human albumin and ACD demonstrated a small but not significant difference from that of 50% homologous plasma/50% saline. As seen in Table 6, the mean yield of granulocytes eluted from nylon filters with plasma/saline was 8.08×10^7 and with PBS plus albumin 6.19×10^7. This represents a difference of 23% between the yields obtained with plasma-containing eluent and PBS plus albumin.

3. Phosphate-buffered saline plus bovine albumin

As seen in Table 6, the use of bovine albumin rather than human albumin in non-plasma containing eluent was intermediate between plasma/saline and PBS plus human albumin. The mean yield of granulocytes represented a figure which was 6% less than that obtained with the use of plasma/saline and 21% greater than that obtained with PBS plus human albumin.

Modification of methods for staining smears from the eluate

The use of a variety of staining and washing times using conventional Wright's stain and distilled water never resulted in satisfactory staining of eluates from nylon filters. Quik-stain was investigated for this purpose. Starting with the 10 second staining time (found satisfactory for whole blood smears) and reducing the staining time by one second for each set of experiments, it was found that a 1 second immersion of the smear in Quik-stain followed by a 10 second immersion in distilled water and a 5 second wash under flowing distilled water resulted in smears in which the white blood cells could be readily differentiated.

Whole-body radioautography of guinea pigs infused with fresh or frozen-thawed granulocytes

The two paired studies carried out in this reporting period confirmed the disposition of infused fresh and frozen granulocytes as reported previously (8). As described previously, the cells of each of the guinea pigs given fresh or frozen granulocytes tagged with ^{14}C-DFP were sequestered essentially in the kidney, spleen, lung and liver within 30 minutes after injection (Figures 4a and 4b)

As determined by densitometry (Table 7), fresh cells were sequestered in decreasing concentrations in the spleen, lung, liver, and kidney. There was no evidence of large numbers of circulating cells as measured in the vena caval blood.

In animals given frozen-thawed granulocytes, the highest concentration of cells was found in the lung followed by liver, spleen, and kidney. There was no evidence of large numbers of circulating cells as measured in the vena caval blood. The primary differences between animals receiving fresh or frozen cells were an apparently significant greater deposition of cells in the spleen and liver of the fresh cells as compared with the frozen cells. There was no obvious difference in cell deposition in lungs and kidney.

In the single animal given only only ^{14}C-DFP, the greatest concentration of ^{14}C-DFP was seen in the lung, kidney, and blood with lesser concentration in the liver and spleen (Figure 4c, Table 7). Labeled DFP was seen for the first time in the blood in the control animal. The concentration of ^{14}C-DFP was also greater in the kidney of the control animal than in any of the four animals which received cells. Almost invariably, on the other hand, the concentration of labeled DFP was lower in the spleen, lung, and liver of the control animal than in those receiving either fresh or frozen cells.

Filtration luekopheresis in the guinea pig

Incision of the mid-ventral line above the trachea and exposure of the left common carotid artery was accomplished. The anatomy was slightly different from that of the rat. The vessel was isolated without the need to retract the sternomastoideus muscle, as is helpful in performing the same procedure in rat.

The carotid artery in the guinea pig appeared to be significantly less muscular (and therefore more fragile) than that in comparably sized rats. The artery broke when handled similarly to that of the rat. In the few animals in which successful cannulation was carried out, the blood flow appeared to be somewhat slower than in comparably sized rats. The jugular vein appeared to be somewhat deeper than that in comparably sized rats but could be cannulated.

It is considered that, on the basis of these experiments, the guinea pig is not the ideal animal for studying filtration leukopheresis. The vessels are small and fragile. There is little supporting perivascular tissue. The animals appear to be relatively labile to the effects of Nembutal for anesthesia. In spite of these drawbacks, the experiments do demonstrate that filtration leukopheresis can be studied with the guinea pig although much greater care will be required to maintain the system than is required when using comparably sized rats.

Kinetics of granulocyte increments

In the present group of experiments, two hours of granulopheresis alone resulted in a granulocyte increment which averaged 11,000/mm^3 (Table 8). Where the pheresis was preceded by an injection of PPP one or two hours prior to initiation of the pheresis, the combinations of treatment resulted in granulocyte increments of over 16,000/mm^3. When PPP injection only was the treatment of choice, such treatment resulted in mean increments of granulocyte counts after one, two, three or four hours of approximately 6,000, 18,500, 12,000 and 10,000/mm^3, respectively.

In the group which received PPP two hours before initiation of two hours of pheresis, an increment in the granulocyte count of over 18,000/mm^3 was observed which did not increase further during filtration. In fact, the granulocyte count in three of the four animals in this group fell during pheresis from the high count reached following PPP injection. This was not true of the animals pheresed one hour after PPP injection and whose count increased during filtration.

It is important to note that the granulocyte count two hours after injection of PPP averaged 22,311/mm^3 while that seen one hour following such an injection was only 9,171/mm^3. It is also important to note that the average maximum granulocyte count following treatment with PPP one hour before two hours of pheresis was 19,612/mm^3 while that following treatment with PPP two hours before a two hour pheresis averaged essentially the same (18,187/mm^3).

DISCUSSION

The studies initiated under this contract, which have subsequently been significantly extended, resulted from an earlier serendipitous finding that plasma from animals which had undergone granulopheresis was capable of improving granulocyte yields when used to pre-treat homologous granulocyte donors (1). There have been several important spinoffs from these original observations. In addition to having significant clinical implications in the area of granulocyte procurement, several studies reported previously to ONR (7,8) have described methods for elucidation of the kinetics of granulopoiesis as well as the whole-body disposition of fresh and freeze preserved granulocytes.

The results described in this report extend those carried out previously. Having worked out optimal dosage regimens for the use of post-pheresis plasma in animals, the next essential information required prior to clinical trials was the determination of the optimal time to pre-treat the donor with PPP. The earlier experiments (1) demonstrated that pre-treatment of rats one hour before initiation of granulopheresis resulted in a doubling of the yield (from 4.3×10^7 to 8.7×10^7 granulocytes per pheresis). With improved methods of pheresis and elution of granulocytes from the filter described in this and in previous reports to the ONR (7,8), we have not only substantiated the phenomenon of increased granulocyte yields following pre-treatment of the donor animals one hour before initiation of pheresis, but we have succeeded in improving the granulocyte yields of both pre-treated and control animals (16.34×10^7 vs. 9.69×10^7). Having now demonstrated that the earlier experiments could not only be repeated but improved upon, the definitive studies to determine the optimal time of pre-treat with PPP were begun.

It was considered that the granulocyte yield probably bears a direct relationship (all other things being equal) to the pre-pheresis granulocyte count of the donor. Since the maximum granulocyte increment in recipients of PPP occurred three hours after injection, it appeared that some time after one hour following treatment with PPP might be optimal for harvesting the greatest number of granulocytes. Acting on that premise, we recently initiated studies in which pre-treatment of donor animals was done two hours before initiation of pheresis. Ultimately, these studies will be extended to include animals pre-treated three hours before initiation of pheresis. Only four experiments which employ two hours pre-treatment have been carried out to this point. Unexpectedly, the yield of granulocytes obtained employing the two hour pre-treatment was not greater, on the average, than that obtained with pre-treatment one hour before pheresis. There are, as yet, too few experiments with two hour pre-treatment to be certain that a longer period between PPP administration and initiation of pheresis will not result, ultimately, in greater yields. However, studies done by others have shown that standard human leukopak filters have a finite "loading" capacity (12). When that capacity has been reached, no further loading is possible. To obtain greater yields of granulocytes, additional filters are added to the system. No studies have been done to date to determine the loading capacity of the miniature filters used in our rat system. In the present studies, in only 2 of 21 experiments has the granulocyte yield from a single filter exceeded 20×10^7 cells. In the 21 experiments carried out prior to the initiation of these studies, none yielded granulocyte numbers in excess of 20×10^7 cells. There is a very real possibility that a level just under 20×10^7 cells is the loading capacity of these filters.

Since each filter is handmade separately, it is reasonable to suppose that, if this is the case, an occasional filter will be packed in such a way that more cells can be harvested from such a filter. This could account for the two filters from which more than 20×10^7 cells were harvested. One must also take into account in these experiments that improper packing can result in a reduced loading capacity. This may account for the experiment in the group pre-treated 2 hours before pheresis in which only 7.70×10^7 cells were harvested. It is obvious that definitive studies of this nature will require investigation of sufficient animals to be certain that results obtained are real.

Another factor which was not taken into account in these preliminary studies was the potency of the post-pheresis plasma. No attempt was made to pair experiments in such a way that the same lot of PPP was used in the one and two hour pre-treatment studies. The reason for this was the small volumes of plasma obtained from the relatively small donors used heretofore. In future experiments, we will use large plasma donors and experiments will be paired appropriately.

It is not surprising that there is a direct correlation between the post-pheresis granulocyte count and the granulocyte yield from the animal. It is obvious that the higher the circulating granulocyte count during pheresis, the more cells come in contact with the nylon fibers per unit of time and, therefore, the greater the opportunity for cells to adhere to the fibers [or to each other, as suggested by others (13)] assuming that the loading capacity has not been exceeded. The importance of establishing that the granulocyte yield is directly proportional to the granulocyte count in the animal is that this is essentially the basis for the hypothesis being put

forth in these studies, i.e., it is reasonable to assume that this is true. However, should a negative correlation be demonstrated, it would cast extreme doubt on all past and future studies of this nature. That the correlation is not absolute is not surprising since there are several variables which ultimately bear upon the final yield of granulocytes eluted from the filter, e.g., variations in filter packing, differences in rate of blood flow from the animal (physiological function), pressure of elution, rate of tapping of the filters, intrinsic errors in WBC counting, determination of WBC differentials, etc. However, we feel that we have demonstrated sufficient sensitivity in this system to provide answers to the questions which we have posed and will pose.

The studies which relate to the correlation between the pre-pheresis granulocyte count and the granulocyte increment attained following two hours of granulopheresis may give some clues regarding the mechanisms of granulopoiesis. The animals used are essentially of the same weight and presumably similar age. One of the three groups demonstrated a significant difference from the other two with regards to granulocyte increments following a standard pheresis. Those with counts below 2,000/mm^3 demonstrated a significantly lower increment in granulocyte count. It would appear that there may be a lower level of endogenous "granulopoietin" in these animals which allowed their normal granulocyte count to be "set" at a lower level. It would also appear that these animals have a smaller capacity to be stimulated to release granulopoietins or at least stimulation of neutrophil mobilizing mechanisms. It would seem therefore, that in those animals with relatively low "normal" granulocyte counts, there is a low titer of endogenuous granulocyte mobilizing factor(s) and only a limited response can be expected in this regard when mobilization of granulocytes is attempted. These observations are pertinent to these studies in that when taken with all other kinds of observations regarding harvesting of granulocytes by this system, one

must take the pre-treatment granulocyte count into account when assessing the results obtained in each animal. It is well known that many bodily humoral mechanisms are affected by the age and sometimes by the weight of an individual. We have chosen to assess the effect of the weight of animals on their ability to mobilize granulocytes during filtration leukopheresis. Intuitively, we are inclined to believe (without documentory evidence) that the differences observed in granulocyte-mobilizing capacity among the four groups of animals are actually age rather than weight related. In terms of understanding basic physiological functions, it would be important to differentiate between the two. However, under the conditions of these experiments, this is not possible. In practical terms, it is important for us to know that animals in the 400-500 gram weight range can be expected to mobilize more granulocytes during a standard pheresis than those of the other three groups. We demonstrated earlier (7) that there is a direct correlation between the granulocyte increment in PPP donors and the ability of that PPP to mobilize granulocytes in recipients of the PPP. The present observations suggest that animals weighing between 400 and 500 grams can be expected to provide the highest titers of granulocyte mobilizing activity as a result of a standard two-hour granulopheresis. This information will be invaluable in selecting future rat donors for production of PPP with high titers of neutrophil releasing factor.

The eluent used for harvesting granulocytes from nylon filters has, in most or all instances, been a plasma-containing medium. We had shown previously (14) that in the rat system, using homologous plasma/saline, the greater the percentage of plasma used (up to 100%) the greater the harvest of granulocytes. It was shown later, however, that for the purposes of these experiments the use of 50% plasma/50% saline was sufficient as a granulocyte eluent. Our aim in these studies was not necessarily to obtain the maximum possible yield but to be able to compare one procedure with another to determine

differences in ability to obtain granulocytes. The cost of the use of even 50% plasma was great, nevertheless. A less expensive alternative was sought. We were certain that there were at least two essential elements in the eluent, i.e., plasma protein and ACD. The latter was required for one or more of the following purposes: (1) acidifying the eluent; (2) chelating calcium; and (3) altering surface charges. Human albumin proved to be an effective replacement for plasma in the eluent. It is easily obtained from commercial sources and is significantly more economical to obtain, in both time and money. However, the cost is not inconsiderable. Dr. Robert Valeri had recently demonstrated that, in certain regards, serum albumin is not species-specific (15). Bovine albumin, a relatively inexpensive material, was later substituted for the human material in the phosphate buffered saline eluent. This proved to be equally effective as a substitute for homologous plasma and further reduced the expense of carrying out these experiments. This is not to suggest that heterologous albumin may be substituted for plasma in clinical studies of this nature, although it may be worthwhile to use homologous albumin as part of the clinical procedure. This remains to be investigated.

Sufficient information of hydroxyethyl starch to replace plasma in this system is not yet available to suggest whether it may be useful in this method. If HES should prove effective in this regard, under the proper conditions, further advantages may accrue in the form of an even less expenseive eluent which will not have the potential for transmitting hepatitis or other blood-borne diseases.

The addition of two additional (and more carefully controlled) pairs of experiments of the disposition of infused fresh and frozen granulocytes has reinforced our earlier impression that this technique is workable and represents a valuable new tool for this purpose.

The two pairs of experiments using fresh or frozen granulocytes were consistent in that blood concentrations of cells were so low as to be undetectable; there were greater concentrations of cells in lung and liver in animals given fresh as opposed to frozen-thawed cells; and there was only a small concentration of cells in the kidney in each of the four animals (two which received fresh and two given frozen-thawed cells). Although there were slight differences between the two pairs of experiments in the disposition of cells in the kidneys, these differences were not considered significant. Further support for the use of this technique for measuring granulocyte disposition was provided by the control experiment. In the animal given only ^{14}C-labeled DFP, significant amounts of radioactivity were measured in the blood. This is reasonable if one assumes a half-life in the blood of at least 30 minutes, which was the period of time between injection of the DFP and sacrifice of the animal. Unlike the experiments in which the DFP was bound to cells, it is reasonable under appropriate circumstances for the DFP to continue to circulate. If infused granulocytes, either fresh or frozen-thawed, are temporarily sequestered in specific organs immediately after transfusion, as we believe they are, one would expect to measure little or no radioactivity in the blood and substantial radioactivity in appropriate organs, i.e., liver, lungs, and spleen. This is, in fact, what occurred. It is true that these organs also showed measurable radioactivity in the control animal. However, this is not surprising since these are very vascular organs and the amount of blood per unit of mass is high. It is important to note that the concentration of radioactivity in the organs was, in general, substantially lower than that in those animals which received granulocyte transfusions. The radioactivity seen in the organs of the control animal may be considered background. The kidney was the only organ which demonstrated greater radioactivity in the control animal than in the test animals. This may further substantiate binding of the DFP to the infused cells.

Bound DFP was not being excreted rapidly through the kidneys while the unbound material was apparently being excreted in substantial amounts during the first 30 minutes after injection.

While additional studies will be required to provide definitive information with regard to the handling of transfused granulocytes by the body, these preliminary studies strongly suggest the method of whole-body radioautography as an important new tool for studies of this nature.

The studies involving combinations of modalities for increasing the granulocyte count of normal rats are especially intriguing. The granulocyte increments ($\bar{x}$ = 11,000/mm^3) obtained following two hours of filtration leukopheresis are almost identical to those obtained previously (3). In the present experiments, we have also shown substantial increments in the granulocyte count of animals from two to four hours after injection of post-pheresis plasma. It is interesting that the combination of injection of PPP followed by filtration leukopheresis was not, in general, capable of mobilizing more than 20,000/mm^3 of granulocytes in the circulating blood. Although we are not yet certain why this is so, certain hypotheses can be suggested. It may be that the kinetics of granulopoiesis combined with a constant removal of granulocytes during filtration will allow mobilization of granulocytes in numbers sufficient to maintain granulocyte levels no greater than about 20,000/mm^3 under the most optimal conditions. We do know that granulocyte counts much in excess of 20,000/mm^3 can be maintained following injection of PPP in the absence of concomitant removal in individual animals (3). A second hypothesis suggests that continued mobilization of granulocytes will occur under the conditions of these experiments only when a circulating granulocyte level - approaching 20,000/mm^3 - is maintained. Continued investigations of granulocyte kinetics will continue to determine which, if either, of these hypothesis is tenable.

CONCLUSIONS

The optimal time for pre-treating donor rats with post-pheresis plasma to improve granulocyte procurement using filtration leukopheresis may be one hour pre-pheresis. This is suggested by incomplete studies involving various pre-treatment schedules. Since these studies are incomplete, we are not yet able to make a definite statement in this regard. If subsequent studies bear this out, a maximum rate of granulocyte mobilization may be the limiting factor. Pre-treatment with PPP less than one hour before pheresis will not yield optimal granulocyte harvests. Granulocyte harvest depends directly on the donor granulocyte count, i.e., the higher the donor count, the greater the yield. Low granulocyte counts in potential granulocyte donors, in general, predict a significantly smaller increment in the granulocyte count during pheresis. These studies suggest that the reason for the low counts in these donors is a low endogenous availability of neutrophil releasing factor. Older animals appear to possess a greater capacity for granulocyte mobilization per unit of body mass.

We have successfully replaced plasma with albumin as the major active material in the eluent for harvesting granulocytes from nylon filters and have been able to modify the staining procedures of blood films low in protein media. This had been an important problem heretofore. We have demonstrated the dispostion of infused fresh and frozen granulocytes in normal guinea pigs using the technique of whole-body radioautography. Using non-cell bound DFP, we have reinforced our impressions that the technique is suitable for this purpose. We have demonstrated that filtration leukopheresis can be carried out in the guinea pig. Analysis of a series of experiments has suggested the limits of granulocyte mobilization.

SIGNIFICANT ACCOMPLISHMENTS

We have provided additional information regarding the potential use of the neutrophil releasing activity of plasma obtained from granulocyte donors. Hopefully this information will be incorporated with other data, previously gathered, with that yet to be obtained to suggest optimal clinical studies.

We have improved the practicality of the rat model for filtration leukopheresis by demonstrating the usefulness of a non-plasma containing eluent, as well as by developing a quick and simple method for assessing the granulocyte harvest. We have demonstrated that whole-body radioautography is a valuable new tool for assessing disposition of infused granulocytes as well as demonstrating the usefulness (and limitations) of the guinea pig for studies of filtration leukopheresis. Basic observations of the kinetics of granulopoiesis have shed additional light on physiological mechanisms of granulocyte mobilization.

The following papers have been published, presented, or accepted for publication:

1. Roy, A., and Ramirez, M. Increased granulocyte counts in recipients of plasma from leukopheresed rats: A dose response study. Experimental Hematology 6:375, 1978.

2. Roy, A. Methods for assaying viability of frozen-thawed granulocytes. Cryobiology 15:232, 1978.

3. Roy, A., Ramirez, M., and Valeri, C. R. Stability of neutrophil-releasing activity of plasma obtained from leukopheresed rats and stored at 4^{o}C. Transfusion 18:734, 1978.

4. Roy, A. Granulopoietic activity and physiologic activity of post-leukopheresis plasma. Accepted for presentation at the annual meeting, Society for Cryobiology, 1979.

5. Roy, A. Trends in American blood banking. Presented at the annual meeting, Bermuda Medical Society, November 13-17, 1978.

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15. Valeri, C. R., Personal Communication, 1978.

TABLE 1

PRE-TREATMENT WITH POST-PHERESIS PLASMA
OF RAT DONORS UNDERGOING FILTRATION LEUKOPHERESIS

	Yield of Granulocytes x 10^7		
Pheresis Alone	PPP 1 Hour Before Pheresis	PPP 2 Hours Before Pheresis	Poor or No Flow (Controls)
5.13	19.49	15.23	0.88
23.44	20.00	7.70	0.89
7.69	8.03	19.96	0.27
10.04	7.91	19.33	0.66
7.76	6.94		0.18
12.97	16.06		0.15
3.73	15.85		
6.73	16.80		
	35.21		
9.69	16.34	15.53	0.51 ← Mean

TABLE 2

CORRELATION OF DONOR POST-PHERESIS

GRANULOCYTE COUNTS WITH GRANULOCYTE YIELDS

Number of Experiments	Post-Pheresis Granulocyte Count x 10^3	Mean Granulocyte Yield x 10^7
1	<5	2.34
14	5-15	7.52
12	15.1-25	11.44
6	>25	17.17

TABLE 3

PRE-PHERESIS GRANULOCYTE COUNT VS POST-PHERESIS INCREMENT

Granulocyte Level/mm^3	Number of Experiments	Mean Initial Gran. Count/mm^3	Mean Increment in Gran. Count/mm^3
<2,000	12	1,130	9,189
2,000-3,999	22	3,194	14,556
≥4,000	24	6,069	14,808

TABLE 4

INCREMENT OF GRANULOCYTE COUNT

VS DONOR WEIGHT

Weight (grams)	Number of Animals	Mean Increment Granulocyte Count
<300	5	4,789
300-399	17	10,963
400-499	19	17,413
≥500	29	12,272

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	-0.89	-12
	12.90	14.46	+1.56	+12
Mean	8.28	7.88	-0.40	-5

TABLE 6

PLASMA/SALINE VS PBS + ALBUMIN FOR ELUTING GRANULOCYTES

50% Plasma/Saline		PBS + Bovine Albumin		PBS + Human Albumin	
Expt. No.	Yield Granulocytes x 10^7	Expt. No.	Yield Granulocytes x 10^7	Expt. No.	Yield Granulocytes x 10^7
1	8.06	1	5.88	1	3.59
2	9.93	2	5.13	2	7.16
3	4.01	3	7.69	3	6.87
4	6.50	4	10.04	4	4.29
5	12.90	5	7.76	5	15.21
6	6.27	6	12.97	6	5.91
7	8.30	7	3.73	7	5.89
8	4.75	8	6.73	8	9.49
9	5.64			9	5.39
10	14.46			10	1.96
				11	2.34
Mean	8.08		7.49		6.19

TABLE 7

OPTICAL DENSITIES ON RADIOAUTOGRAMS OF SELECTED GUINEA PIG TISSUES FOLLOWING INFUSION OF FRESH OR FROZEN-THAWED ^{14}C-DFP-LABELED GRANULOCYTES OR ^{14}C-DFP ALONE

Expt. No.	Total ^{14}C-Labeled Gran. Infused x 10^3	Granulocyte Preparation	Exposure Time (days)	Optical Density (x 10^{-2})* Blood	Kidney	Spleen	Lung	Liver
2	150	Fresh	43	0	1	28	14	13
	150	Frozen-thawed	43	0	4	3	8	7
3	325	Fresh	82	0	2	-	21	9
	325	Frozen-thawed	82	0	1	6	13	6
4	350	Control (^{14}C-DFP only)	82	8	8	2	9	3

*Corrected for background.

TABLE 8

EFFECT OF GRANULOPHERESIS, INJECTION OF PPP, OR A COMBINATION OF THESE ON THE GRANULOCYTE INCREMENT IN NORMAL RATS

Number of Animals	Treatment	Timing of Granulocyte Count	Mean Granulocyte Increment After Total Treatment/mm^3
18	2 hr pheresis	After pheresis	11,000
9	PPP injection	1 hr after injection	6,047
8	PPP injection	2 hr after injection	18,558
8	PPP injection	3 hr after injection	12,000
8	PPP injection	4 hr after injection	10,000
9	2 hr pheresis, 1 hr after PPP injection	After pheresis (3 hr after PPP injection)	16,600
4	2 hr pheresis, 2 hr after PPP injection	After pheresis (4 hr after PPP injection)	16,404

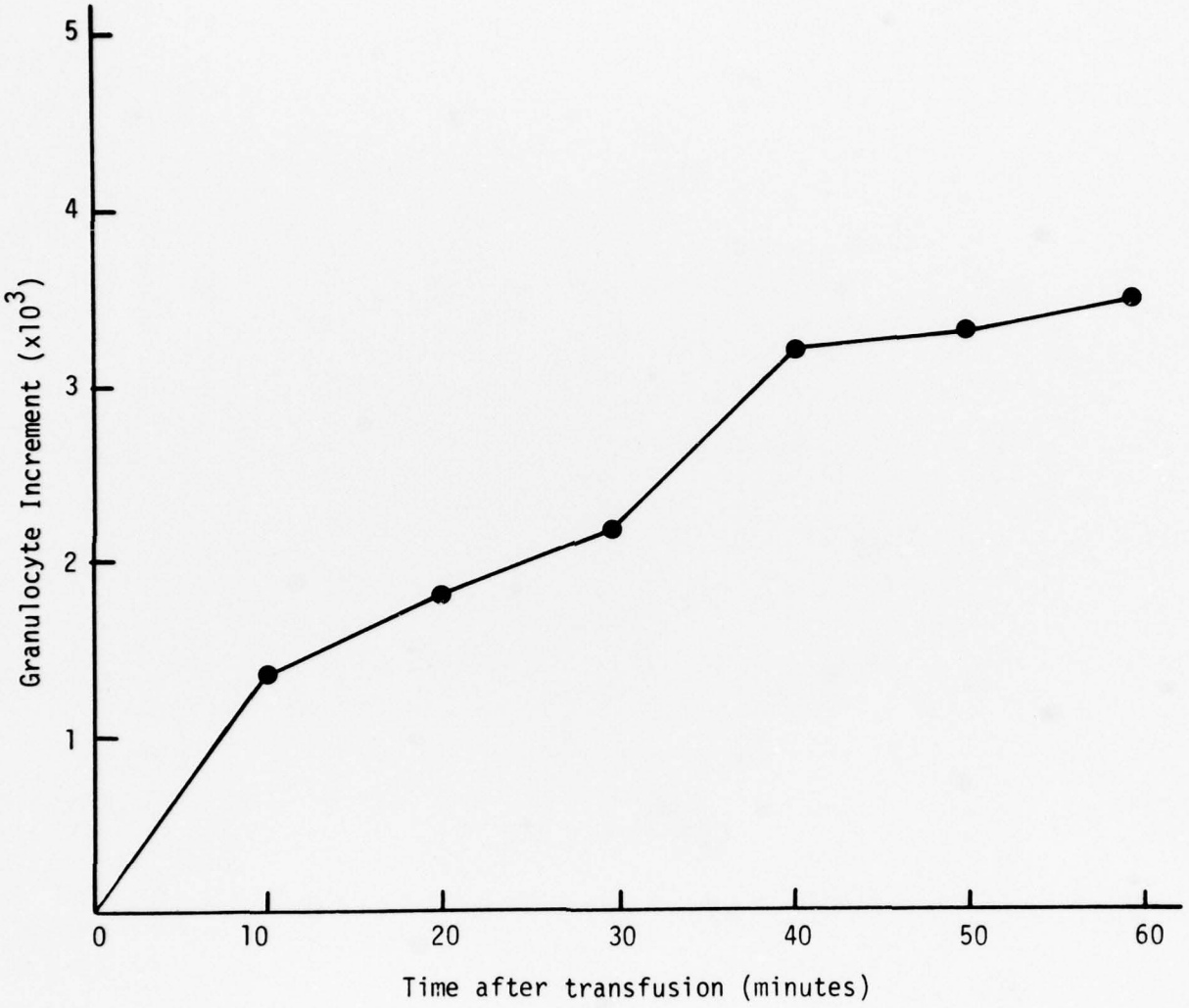

Figure 1

MEAN GRANULOCYTE INCREMENTS IN NORMAL RATS GIVEN AN INTRAVENOUS INJECTION OF PPP

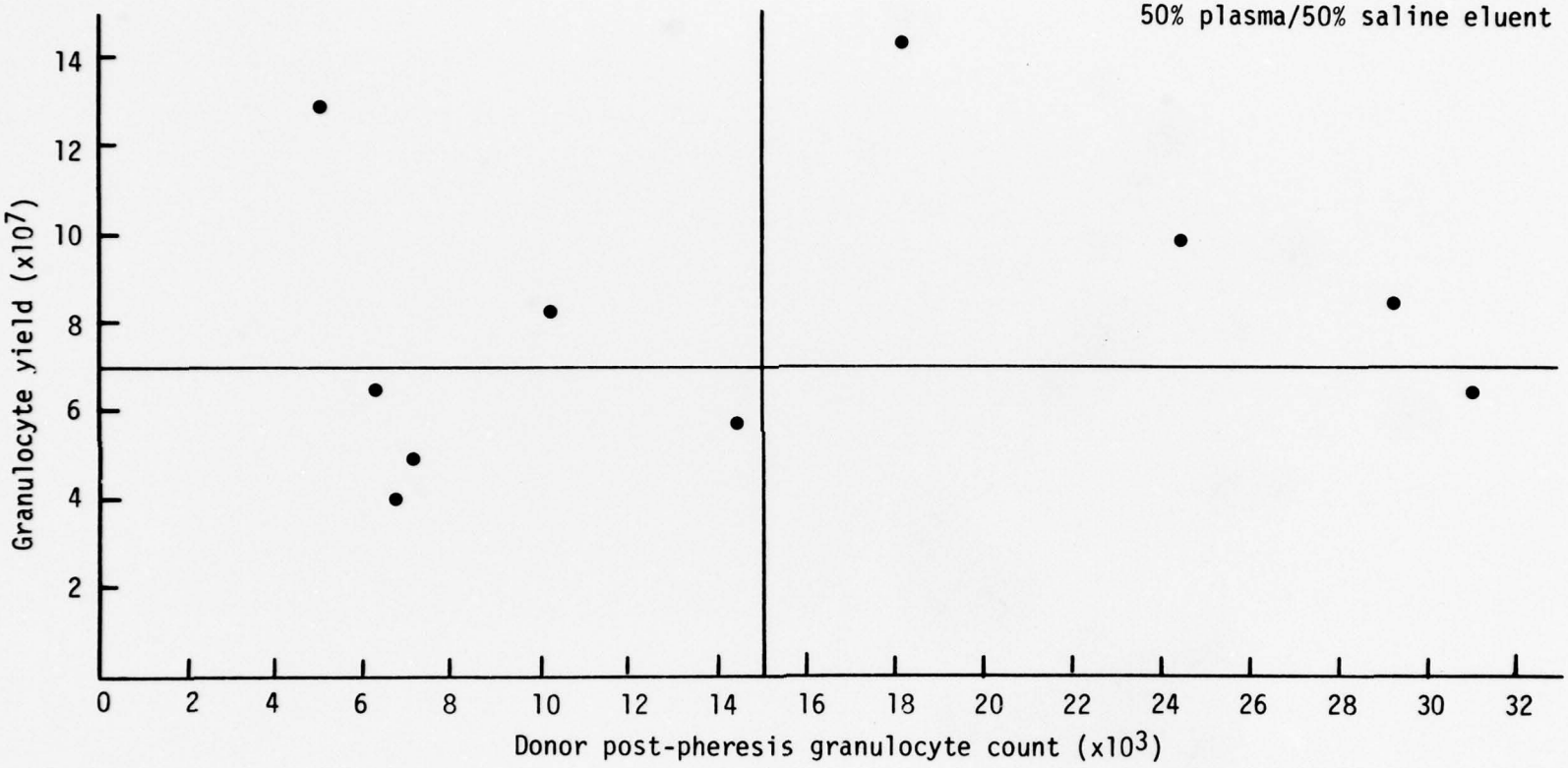

Figure 2

CORRELATION BETWEEN DONOR POST-PHERESIS GRANULOCYTE COUNT AND GRANULOCYTE YIELD

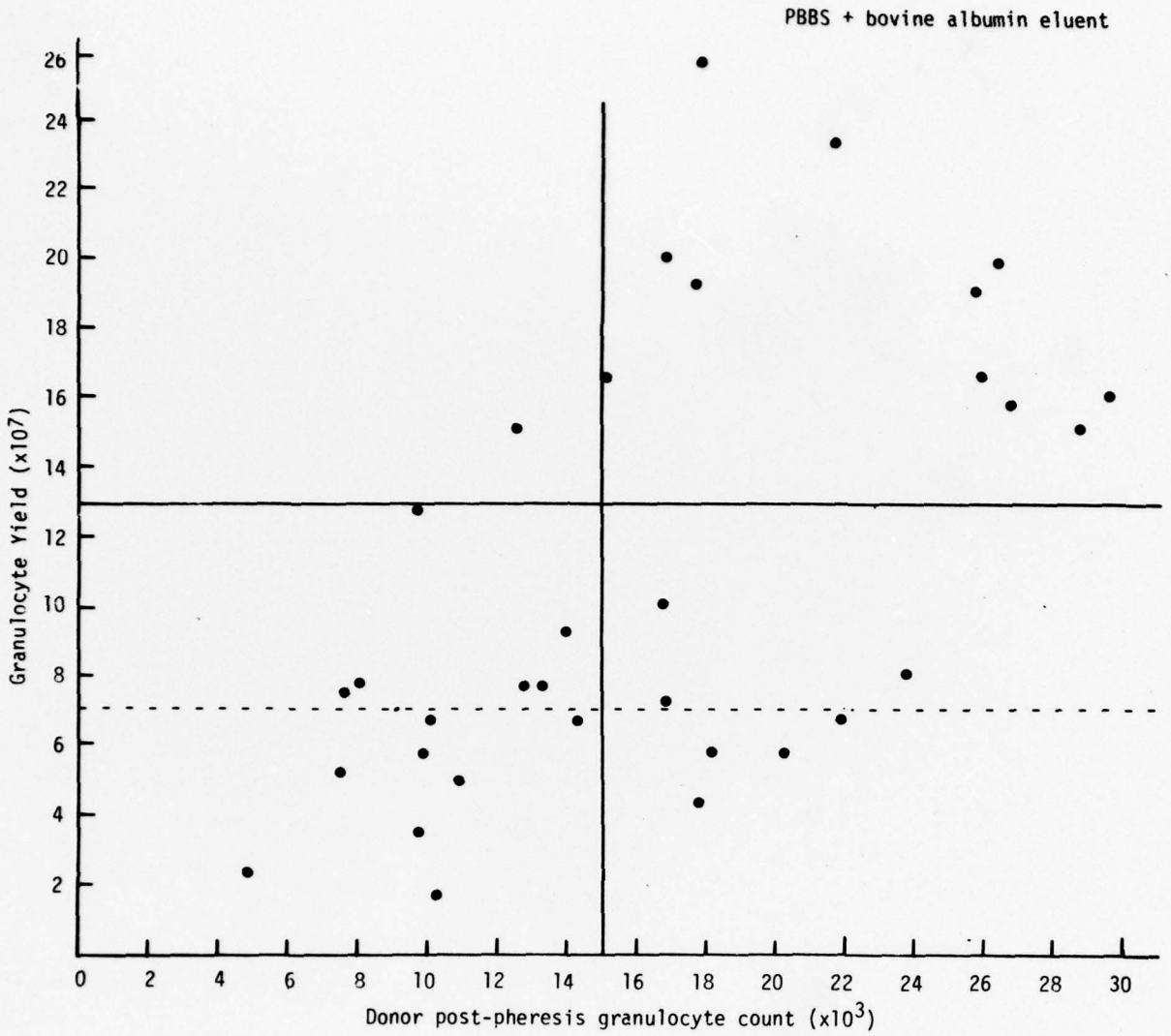

Figure 3

CORRELATION BETWEEN DONOR POST-PHERESIS GRANULOCYTE COUNT AND GRANULOCYTE YIELD

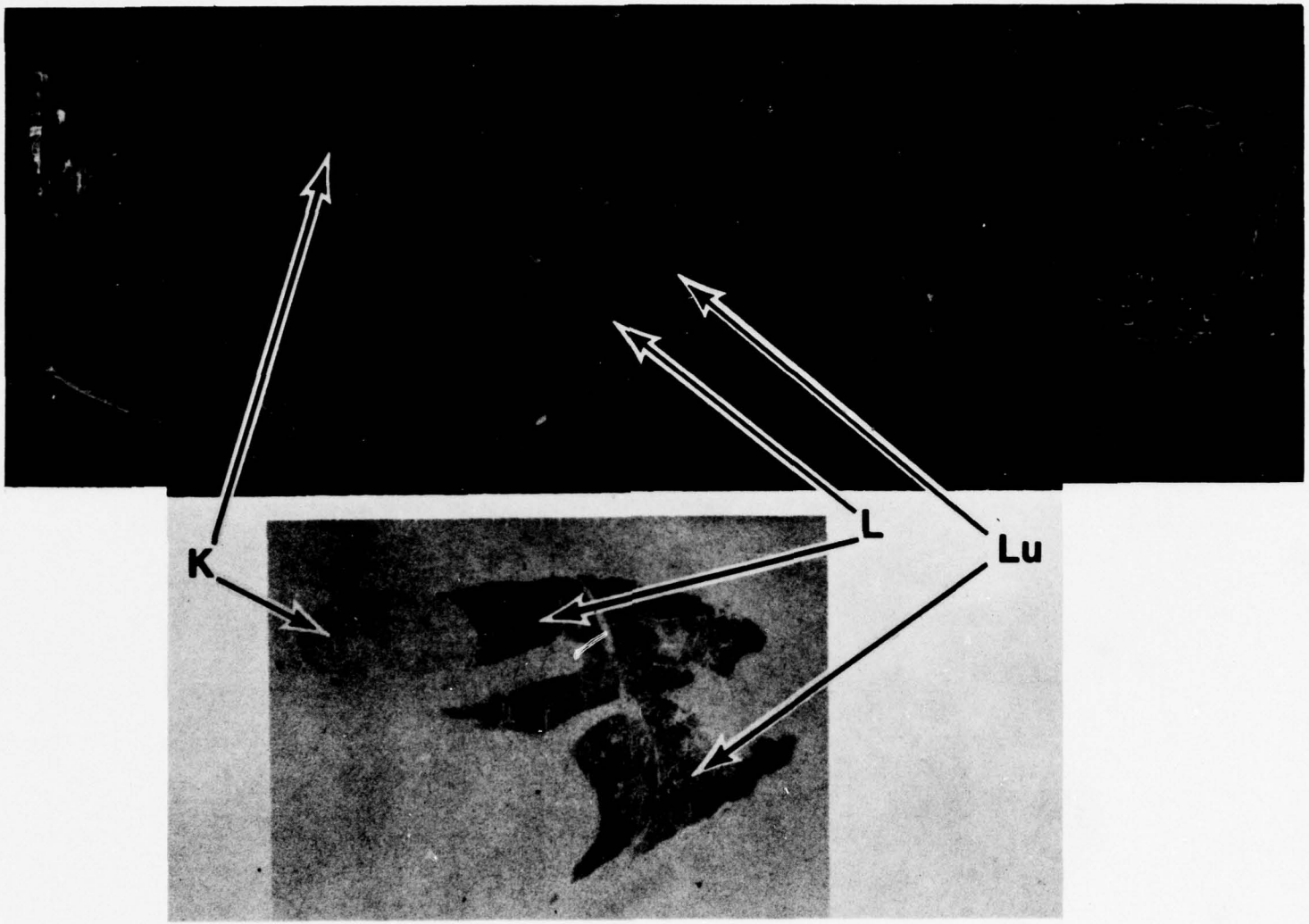

Figure 4a Whole-body section (above) and corresponding whole-body radioautogram (below) of guinea pig transfused with fresh guinea pig granulocytes tagged with radiolabeled DFP. Labeled granulocytes are seen in the kidney (K), liver (L), and lungs (Lu). No statement can be made regarding the spleen, since the spleen does not appear in this particular plane of section.

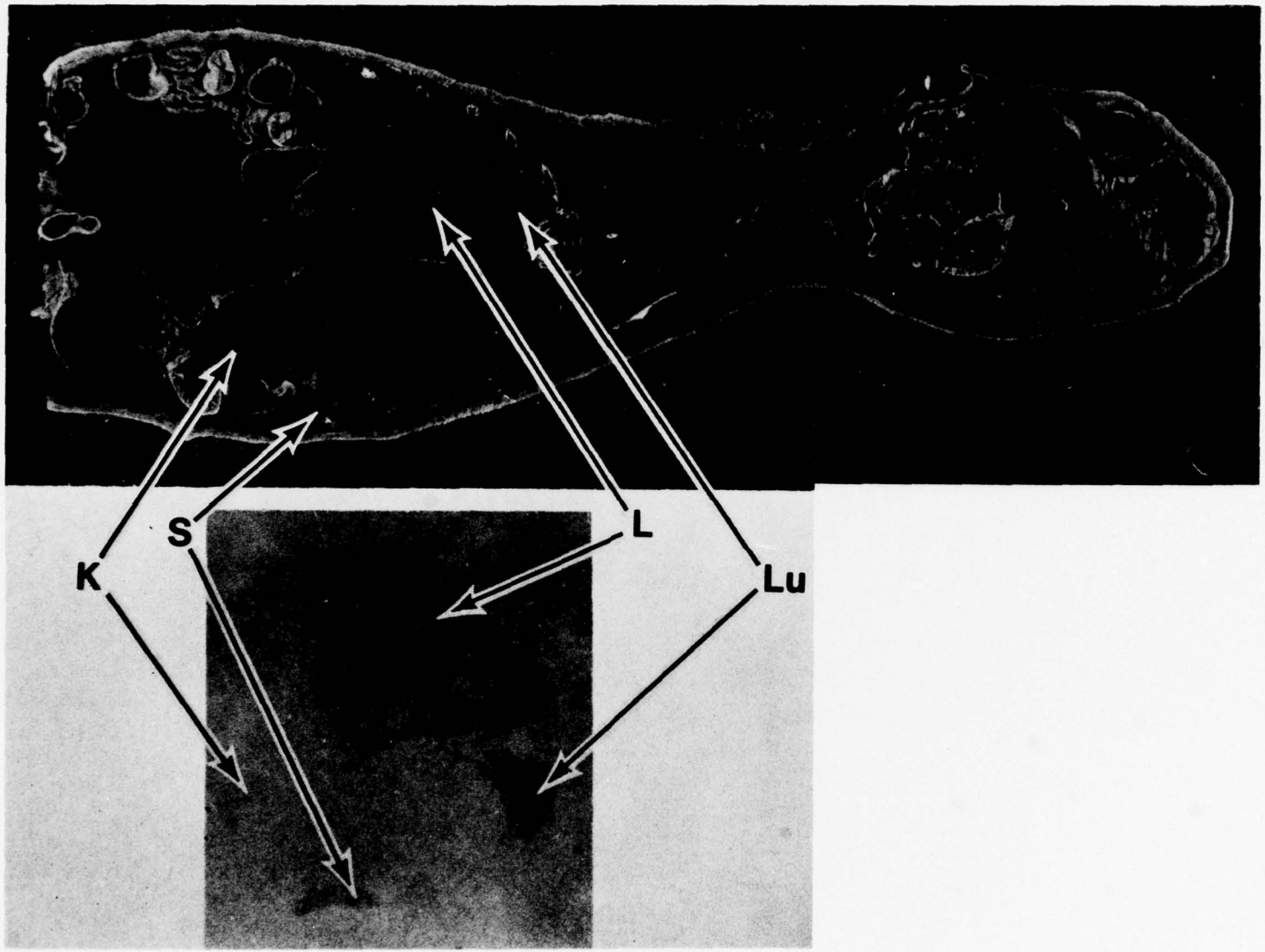

Figure 4b Whole-body section (above) and corresponding whole-body radioautogram (below) of guinea pig transfused with frozen-thawed guinea pig granulocytes tagged with radiolabeled DFP. Labeled granulocytes are seen in the kidney (K), spleen (S), liver (L), and lungs (Lu).

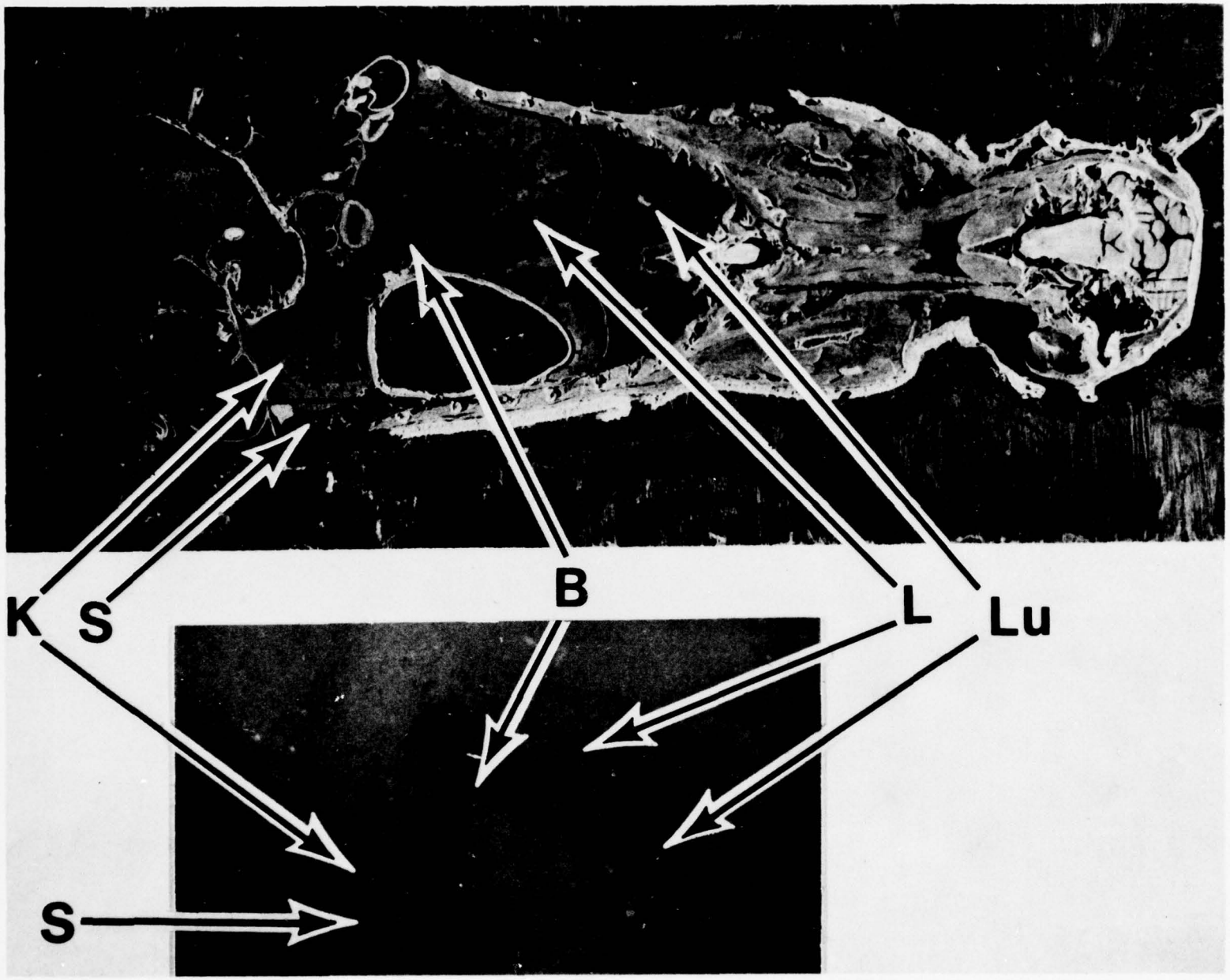

Figure 4c. Whole-body section (above) and corresponding whole-body radioautogram (below) of guinea pig transfused with radiolabeled DFP (^{14}C-DFP). Labeled DFP is seen in the blood (B), kidney (K), spleen (S), liver (L), and lungs (Lu).

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leukopheresis, filtration leukopheresis, granulocyte kinetics, neutrophil-releasing activity, neutrophil-releasing factor

10 to the 9th power

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

These studies are a continuation of those previously reported to ONR. The studies were the result of a serendipitous observation that rat or human granulocyte donors who had undergone filtration leukopheresis demonstrated a granulocytosis in spite of the removal of up to 1 x 10^9 granulocytes. Subsequent studies suggested the clinical usefulness of the use of plasma obtained from a granulocyte donor (PPP) to improve granulocyte yields following subsequent donations.

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20. Abstract (contd)

Previous tasks under this contract elucidated effective dose levels of PPP to obtain increased granulocyte harvests as well as several studies to determine the ideal donor and kinetics of granulocyte mobilization. The present studies, some of which are incomplete at this writing, support the previous observations that pre-treatment of rat granulocyte donors at least one hour before pheresis will indeed improve granulocyte yields. It was suggested that pre-treatment at less than one hour before pheresis would not improve the yield. Studies are still in progress to determine whether there is any advantage in prolonging the interval between PPP injection and initiation of granulopheresis. We have demonstrated that heterologous albumin (bovine or human in the rat) can effectively replace homologous plasma for elution of granulocytes from nylon filters. Additional studies demonstrated that a low initial donor granulocyte level would generally portend a poor granulocyte increment during pheresis and this probably resulted from low levels of endogenous granulocytopoietins in young animals. Additionally, we have demonstrated with carefully controlled experiments the use of whole-body radioautography to show organ distribution of fresh and frozen granulocytes. ~~These studies also included the use of densitometry to quantitate the findings.~~ A series of experiments was carried out which suggested that approximately 20,000 granulocytes per mm^3 was the limit to which granulocyte mobilization in the peripheral blood could be pushed while granulocytes were simultaneously being removed in large numbers by filtration leukopheresis.

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