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A functional comparison of ^{111}indium-labelled elicited peripheral blood neutrophils and peritoneal neutrophils in the rat

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SUMMARY

A functional comparison between elicited peripheral blood neutrophils has been made *in vivo* and *in vitro*. Preliminary experiments showed that separation of peripheral blood cells on a metrizamide gradient yielded too few neutrophils for efficient radiolabelling with indium (In): hence a mixed cell preparation comprising 80% neutrophils was elicited in the peripheral blood of adult male rats by the administration of endotoxin (0·25 mg i.a.) and cobra venom factor (200 μl i.p.) 20 h before. Peritoneal neutrophils were collected 4 h after the i.p. injection of 6 ml thioglycollate. Both populations differed markedly from normal peripheral neutrophils on the *in vitro* testing of random locomotion, chemotaxis and phagocytosis of Candida. After labelling with ^{111}In-tropolonate, a greater proportion (mean = 8%) of peripheral blood cells localized to an *E. coli*/Freund's complete adjuvant-induced abscess compared with peritoneal neutrophils (mean = 3%). The abscess could be visualized externally by scanning with both cell preparations, but the distribution of activity differed markedly. The greater hepatic sequestration of peritoneal neutrophils sugggested cell damage or activation. To overcome the difficulty of harvesting normal peripheral blood neutrophils in the rat, either of these populations can be used to follow the kinetics of inflammation. However, elicited peripheral blood cells yield a higher proportion of responding cells.

Keywords Indium neutrophil labelling abscess

INTRODUCTION

Our knowledge of neutrophil kinetics in the acute inflammatory response in man has been considerably advanced by the use of radiolabelled leucocytes (Peters *et al.*, 1983; Saverymuttu *et al.*, 1983). Animal studies so far have been confined to the larger laboratory animals such as dogs (Thakur, Coleman & Welch, 1977; McAfee *et al.*, 1980), baboons (Vecchione *et al.*, 1984) and rabbits (Lane *et al.* 1982) which are limited in their usefulness because of expense and availability. No previous studies of localization of labelled peripheral blood neutrophils have been possible in the rat because of the animals' small blood volume and low neutrophil:lymphocyte ratio. Furthermore while a pure neutrophil preparation harvested from the peripheral blood should ideally be used, any density gradient techniques used for enrichment of cell fractions will alter *in vitro* kinetics (Saverymuttu *et al.*, 1983). On the other hand, pure preparations of peritoneal neutrophils can be elicited by the injection of casein and glycogen, and then radiolabelled; these

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have been shown to localise to a site of inflammation (Perper *et al.*, 1974; Jones, Richardson & Kay, 1977).

We report here a method for collecting rat peripheral blood neutrophils by inducing a neutrophilia, using endotoxin and cobra venom factor (CoVF). This combination produces both an increase in the total white cell count and a relative increase in neutrophils at 24 h. After sedimentation to remove contaminating red cells, a mixed preparation of peripheral blood leucocytes, labelled with ^{111}indium was injected into rats with artificially created abscesses. A comparison with elicited peritoneal cells was made in terms of *in vitro* function tests; localization to the abscess and organ distribution determined by gamma camera imaging; and by the uptake in individual organs at the time of sacrifice.

MATERIALS AND METHODS

Animals. An inbred strain of male PVG rats weighing 150 g were supplied by Olac UK.

Peritoneal neutrophils. Six millilitres of fluid thioglycollate medium (DIFCO Laboratory) was injected i.p. Four hours later the animals were sacrificed and the peritoneal cells collected by lavage into 18 ml of phosphate-buffered saline (PBS) with 2 ml ACD (NIH formula A). The cell suspension was washed once and resuspended in PBS/fetal calf serum (FCS).

Peripheral blood neutrophils. Endotoxin (Sigma Chemical Company) 0·25 mg. i.a. and CoVF 200 μl i.p. (prepared by Mr Alan Dash, using a technique described by Pepys, Tompkins & Smith, 1979) were injected 20 h before harvesting. The animals were exsanguinated by intra-cardiac puncture into ACD; the blood was mixed with heated starch (Plasmasteril, Fresenius) in a ratio of blood:starch = 6:1; and allowed to sediment for 1 h. The supernatant containing white blood cells was washed once in PBS/FCS at 90*g*.

Neutrophil studies. All cell counts were performed in a Coulter counter (Coulter Electronics). Differential cell counts were made on May–Grunwald–Giemsa smears. Trypan blue exclusion tests were performed to assess viability.

A functional comparison of normal neutrophils with elicited peritoneal and peripheral neutrophils was made using previously described tests of chemotaxis, random locomotion and *Candida guilliermondii* phagocytosis and killing (Jones *et al.*, 1983), on unlabelled cells maintained in rat plasma, and compared with normal cells.

Neutrophil labelling. Cells were resuspended in 0·5 ml plasma to which 300 μCi ^{111}In in 0·01 M HCl with 0·05 ml 10^{-4} M troponolone was added. After 5 min incubation, the cells were washed with 15 ml. PBS/FCS at 90*g* for 5 min, and then resuspended in PBS/FCS.

Layering 1 ml of the labelled mixed cell preparation from the peripheral blood onto a 65%/80% Percoll (Pharmacia)/plasma gradient, and centrifugation at 150*g* for 20 min resulted in a relative separation of contaminating platelet clumps, lymphocytes, and RBC from neutrophils, as determined by May–Grunwald–Giemsa stain. This technique enabled an estimation of radioactivity specifically associated with the neutrophils to be made.

Concentrations of contaminating endotoxin were estimated by the modified Limulus assay (Webster, 1980).

Abscesses. Abscesses were induced by the s.c. injection of 10^7 heat killed *E. coli* suspended in 0·5 ml PBS/0·5 ml Freund's Complete adjuvant (FCA), into the shaved right hind-quarters of the experimental animal. A control injection of 0·5 ml PBS was injected into the opposite hind-quarter.

Imaging. The labelled cell preparations were injected immediately after preparation, 1 h after the creation of the abscess, into the tail artery, with the animal positioned beneath a gamma camera (IGE 400T). Images were taken of the site of inflammation at 3·5 h, with the animal prone and supine, in order to record the relative activity in the liver, lungs and spleen.

Organ localization at the time of sacrifice. Animals were sacrificed at 4·5 h and lung, spleen, a segment of liver, the abscess, control PBS site and a section of normal skin were weighed and counted in a Packard gamma-counter. Total counts for the inflammatory sites and organs were calculated.

Transferrin control. In order to estimate the contribution of cell free ^{111}In associated with

transferrin, in the activity attributed to the abscess, transferrin was labelled with ^{111}In by incubating cell free plasma with $^{111}In\ Cl_3$. After i.a. injection, scans were performed at 3·5 h. Activity associated with the abscess, control PBS site, skin and individual organs was estimated at 4·5 h in the same way as for the experimental animals.

Platelet control. To examine the possible contribution of contaminating labelled platelets, to the measured abscess radioactivity following administration of the mixed leucocyte preparation, a platelet fraction was labelled with ^{111}In and injected into an animal with an induced abscess.

Platelet labelling. An untreated animal was exsanguinated by intra-cardiac puncture into the ACD; the blood was mixed with heated starch in a ratio of blood : heated starch 6 : 1, and allowed to sediment for 1 h. The supernatant containing leucocytes and platelets, was made up to a volume of 15 ml with PBS/FCS and centrifuged at 100*g* for 5 min. Further ACD (1 ml per 10 ml supernatant) was added, and the resulting preparation was centrifuged at 200*g* for 10 min to obtained a platelet pellet, that was labelled with ^{111}In using the same technique as described earlier for neutrophils.

RESULTS

Peritoneal neutrophils

Eighty million neutrophils were extracted from the peritoneal cavity of animals injected with thioglycollate; $4{\cdot}3 \times 10^6$ neutrophils with $11{\cdot}0 \times 10^6$ ct/min injected i.a. into each of four animals. The preparations contained only contaminating red blood cells in the ratio of neutrophils: RBC = 100 : 120. Free activity was 8%. Labelling efficiency was 30%. Viability, as determined by dye exclusion test was 98%.

Peripheral blood neutrophils

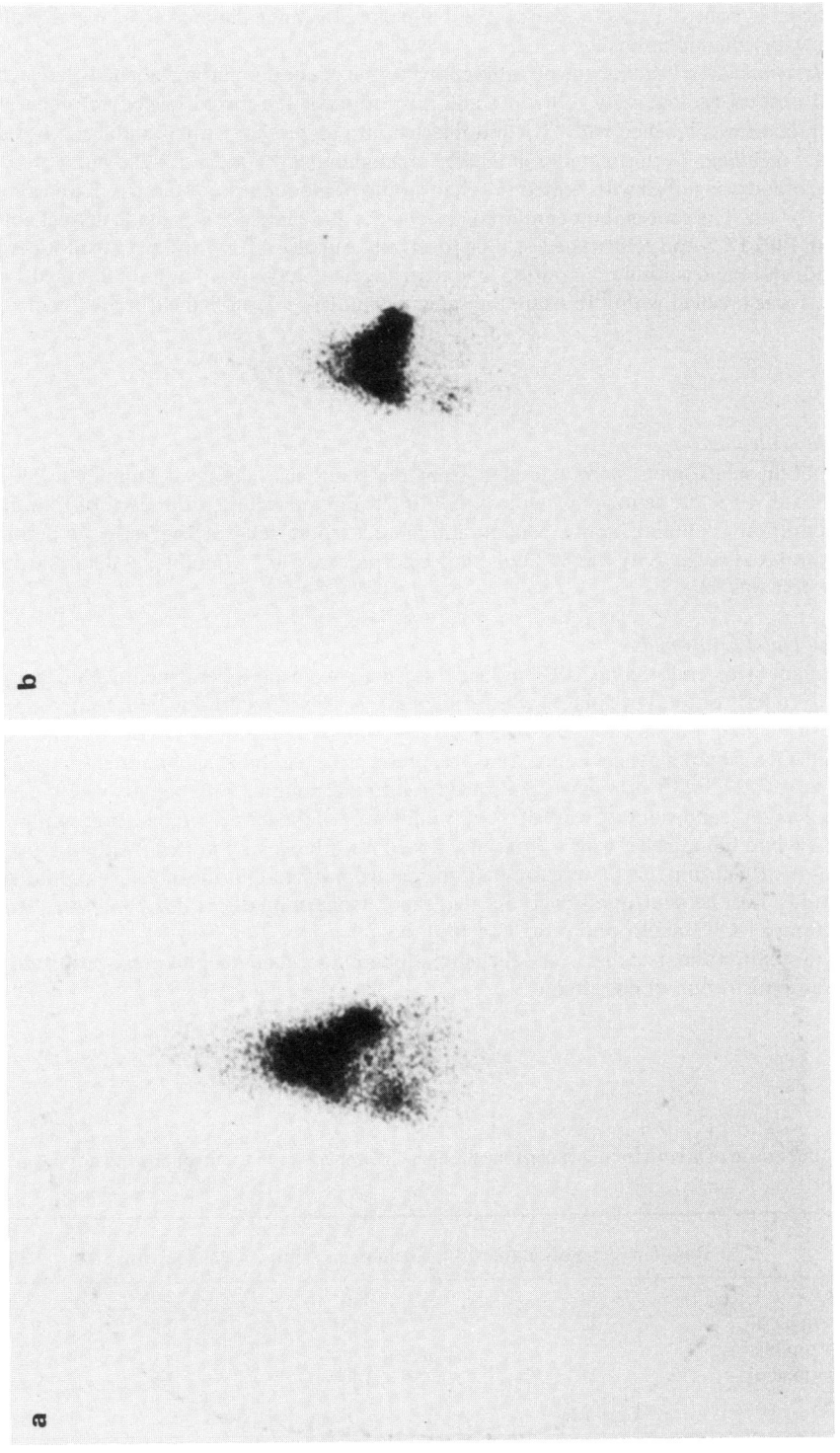
Pre-treatment with endotoxin/CoVF induced an increase in peripheral neutrophil count from about 30% to 95% of total peripheral white cell count. After sedimentation with heated starch and labelling, the final preparation consisted of neutrophils : lymphocytes : RBC in the ratio of 134 : 8 : 100. Occasional platelet clumps were also present. Two million, six hundred thousand white blood cells, with $21{\cdot}3 \times 10^6$ ct/min were injected i.a. into each animal. Free activity was 9%; labelling efficiency was 25%; and viability was 93%. One millilitre of a labelled cell preparation containing 10^6 peripheral white blood cells, with a total of $7{\cdot}3 \times 10^6$ ct/min was separated on the Percoll saline gradient $4{\cdot}5 \times 10^6$ ct/min (62%) were found in the neutrophil band (at the interface), although only $3{\cdot}1 \times 10^6$ (44%) of the neutrophils were located there. Contaminants in this band were RBC and platelet clumps (RBC : neutrophil ratio = 14 : 100).

No endotoxin was detectable in the elicited peripheral blood neutrophil preparation, 20 h after systemic administration of endotoxin.

Table 1. Comparison of *in vitro* testing of peritoneal, elicited peripheral blood neutrophils and normal peripheral neutrophils

	Random locomotion (mm)	Chemotaxis (mm)	Phagocytosis (%)	Killing (%)
Peritoneal	34	114*	77*	90
Elicited peripheral blood neutrophils	22	113*	88*	90
Normal peripheral neutrophils	23	40	65	87

* Significant difference from normal cells.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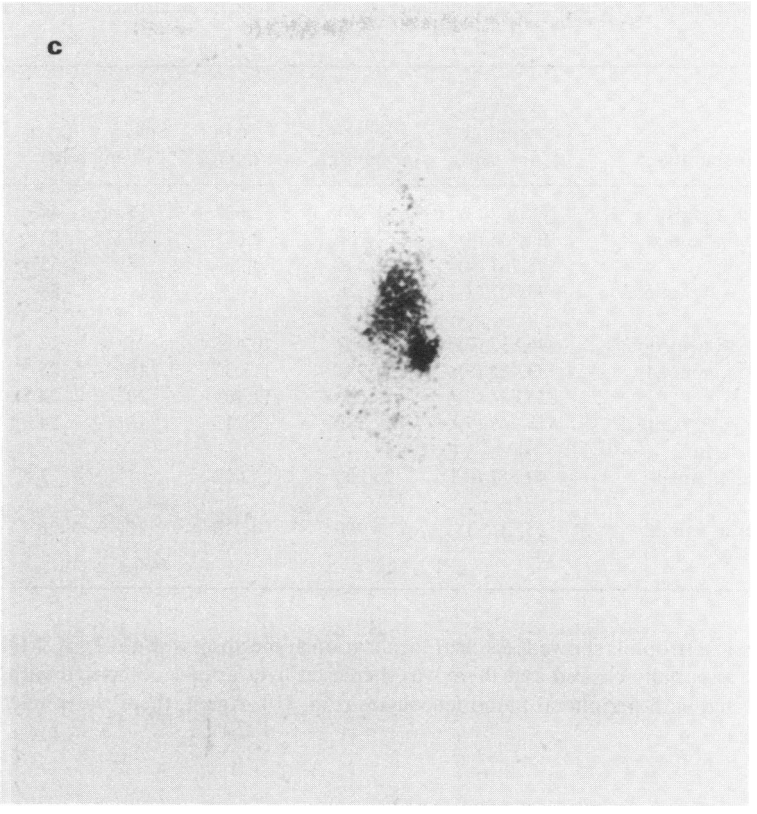

Fig. 1. Scans at 3·5 h—comparison of ^{111}In neutrophils elicited in peripheral blood and peritoneum, showing localization of abscess site and organ distribution. (a) Anterior scan 3·5 h after return of ^{111}In-labelled peripheral blood leucocytes showing in addition to the normal activity in the spleen (right), liver (left) and blood pool in the lung, abnormal activity can be clearly seen in the induced abscess (bottom left). No activity can be seen in the control injection site on the right. (b) Anterior scan 3·5 h after return of ^{111}In-labelled peritoneal leucocytes showing heavy liver uptake (top left), normal splenic uptake (top right), and negligible lung blood pool activity. Faint abnormal activity can be seen in the induced abscess (bottom left). (c) Anterior scan 3·5 h after return of ^{111}In peripheral blood platelets showing normal spleen activity (top right), normal liver activity (top left) and blood pool in the lungs. No activity can be seen in the induced abscess site or control injection sites.

In vitro *neutrophil function*

Elicited peripheral blood and peritoneal neutrophils differed markedly from normal peripheral blood neutrophils on tests of chemotaxis and phagocytosis (Table 1). Peritoneal cells showed also increased random locomotion in micropore filters compared with both elicited and normal peripheral blood neutrophils but both elicited populations showed a nearly three-fold increase in the distance travelled towards casein in a micropore filter chemotaxis assay. Candida phagocytosis, as estimated by the percentage of cells able to ingest one or more targets, was significantly increased in both elicited populations, more so in the elicited peripheral blood cells. Candida killing was not significantly affected, estimating the percentage of cells able to kill at least one target after ingestion.

Imaging

Elicited peripheral blood leucocytes had clearly localized in the abscess site by 3·5 h (Fig. 1a). At this time, lung activity was still high due to a blood pool image. Activity in the spleen was greater than in the liver, while that of the bone marrow was minimal. No activity was evident in the PBS injection sites.

Table 2. Localization of ^{111}In-labelled neutrophils elicited in the peritoneum and from peripheral blood in *E. coli* FCA abscess at 4·5 h

Injected preparation	Abscess (ct/min/g) (% inj. cells)	PBS skin (ct/min/g)	N skin (ct/min/g)	Spleen (% inj. cells)	Liver (% inj. cells)	Lungs (% inj. cells)
Peritoneal neutrophils	168,307 (3)	21,904	2,687	15	44	5
$11{\cdot}0 \times 10^6$ ct/min/animal	195,754 (3)	4,256	2,671	17	51	6
Free activity = 8%	193,761 (4)	23,046	3,237	15	53	6
No. cells = $4{\cdot}3 \times 10^6$/animal	193,907 (3)	12,228	3,055	19	58	8
Elicited						
Peripheral blood neutrophils	946,837 (7)	22,687	10,415	21	22	14
$21{\cdot}3 \times 10^6$ ct/min/animal	1,133,485 (9)	31,931	11,030	10	25	11
Free activity = 9%	855,844 (9)	15,656	11,401	20	24	9
No. cells = $2{\cdot}6 \times 10^6$/animal	858,549 (7)	17,282	10,215	18	24	13
Labelled transferrin						
$6{\cdot}2 \times 10^6$ ct/min/animal	40,552 (0·1)	25,200	25,662	1	1·5	3
Platelets						
$11{\cdot}1 \times 10^6$ ct/min/animal	15,715 (1)	6,700	4,716	10	26	5
Free activity = 3%						

Peritoneal neutrophils showed less clear localization at the abscess site at 3·5 h. At this time lung activity had essentially cleared and there was increased liver uptake compared with that seen in animals injected with peripheral blood leucocytes (Fig. 1b). Again, there was no activity in PBS injection sites.

Localization

Peripheral blood neutrophils showed increased localization to the abscess, with at least 7% of the injected activity recovered in the abscess, compared with labelled peritoneal cells, when at the most 4% of injected activity was found in the abscess (Table 2). Individual organ counting verified the patterns obtained by imaging.

Transferrin and platelet control experiments

^{111}In-labelled transferrin experiments showed little localization of transferrin to the site of the abscess (0·1% of the total activity injected); and the amount of activity localized here was 1·5 times that of the PBS injected skin, or the normal skin (Table 2).

Similarly ^{111}In platelets failed to localise on scanning in the abscesses or in the control injection site. The pattern on scanning was similar to that of peripheral leucocytes with a prominent lung image at 3·5 h and increased spleen activity compared with the liver (Fig. 1c).

DISCUSSION

This study shows that both ^{111}In-labelled CoVF endotoxin elicited peripheral blood neutrophils and thioglycollate-induced peritoneal neutrophils are capable of migrating to experimental abscess in significant numbers to be visualized by gamma scanning. Elicited peripheral blood neutrophils localised to the *E. coli*/FCA-induced abscess in at least 40 times the number that localized in the control site—a similar figure to that found by Issekutz & Movat (1980) using Cr-labelled rabbit neutrophils and formalin killed *E. coli* abscess.

Scanning showed that preparations of peripheral blood leucocytes produced clearer and denser localisation of the abscess than peritoneal neutrophils. By quanitation of individual organs at sacrifice, peripheral blood leucocytes were estimated to be more than twice as effective as peritoneal leucocytes in accumulating at abscess sites.

In vitro tests of neutrophil function showed slight differences between the two preparations, although both differ from unelicited blood neutrophils. However there was a marked difference in *in vivo* distribution with approximately twice as great hepatic sequestration of peritoneal cells compared with peripheral blood cells. The net effect is to reduce the available neutrophil populations available to respond to the trophic stimulus.

A recent study in humans has shown that the kinetics and distribution of ^{111}In-labelled leucocytes are closely related to separation techniques where additional manipulation, particularly gradient separation result in prolonged pulmonary transit, excessive liver uptake and failure to localise in inflammatory sites (Saverymuttu *et al.*, 1983). *In vitro* tests of these human cell populations were not sufficiently sensitive to detect any differences between the cell preparations that produced marked changes *in vivo*. In the rat model, the interpretation of lung activity is more complex. Because of the small size of the rat lung field, the large cardiac pool relative to the anterior surface of the chest and the overlap of liver with lung fields, analysis of lung transit was much more difficult and was not attempted. The lung activity seen on the late 3·5 h scans does not reflect first pass lung transit but rather blood pool activity. Thus the low level of lung activity obtained with peritoneal cells was due to the low blood recovery associated with high liver uptake. Conversely the prominent lung activity seen with peripheral blood cells and platelets reflect high blood recovery and lower liver sequestration.

Thus clear differences exist between the distribution of elicited peripheral cells and peritoneal cells. However we are uncertain how to explain specifically the distinguishing features that account for the different patterns. Either of these populations can be used to study inflammation in the rat, but the elicited peripheral blood neutrophils are more sensitive. How close elicited peripheral neutrophils and peritoneal cells are to normal peripheral neutrophils is not known but *in vitro* studies do show clear differences. From our knowledge of human labelled neutrophils we feel that the pattern seen with peripheral blood leucocytes most closely reflects normal leucocyte kinetics. The studies that use peritoneal cells must be interpretated with caution.

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