THE ORIGIN OF MACROPHAGES FROM BONE MARROW IN THE RAT

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Received for publication May 7, 1964

IT was shown in the previous paper (Volkman and Gowans, 1965) that the macrophages which accumulate on " skin windows " and on subcutaneous coverslips in the rat are derived from cells which emigrate from the blood ; and, further, that the precursors of these blood cells are dividing continuously and at a rapid rate in tissues other than the area of inflammation. In the present work two sets of experiments were carried out in an attempt to identify the tissues in which the precursors of macrophages reside. First, the ability of rats to mobilize newly-formed macrophages was studied after various tissues had been removed, depleted or damaged. Second, normal rats were injected with suspensions of radioactively-labelled cells from various sources to determine which of them could give rise to labelled macrophages on the coverslips.

MATERIALS AND METHODS

Animals.—The donors and recipients of cell suspensions were always members of the same highly inbred strain of rat : either a hooded (HO) or an albino (AO) strain. In other experiments male and female rats belonging to a non-inbred albino colony were used. The animals weighed 100–250 g. at the beginning of each experiment.

Macrophages.—Exudate macrophages on skin windows and on subcutaneous coverslips were obtained from rats by the method of Volkman and Gowans (1964). The great majority of coverslips were removed and examined 24 hr. after their application.

Tritiated thymidine.—Rats bearing coverslips were given a single intravenous dose of 1 μc of tritiated thymidine per g. body weight (Radiochemical Centre, Amersham ; specific activity 4·32–9·5 c/mM). Single or multiple intravenous injections of the same dose were given to other rats in order to label *in vivo* cells and tissues which were later infused as suspensions into recipients bearing coverslips.

Tritiated adenosine.—Thoracic duct lymphocytes were labelled *in vitro* by incubation for 1 hr. at 37° with tritiated adenosine at a concentration of 10 μc./ml. (Schwarz Laboratories Inc. ; specific activity, 1 c./mM). This procedure labelled the ribonucleic acid of small lymphocytes and both the ribo- and deoxyribonucleic acid of the large lymphocytes (Gowans, 1962 ; Gowans and Knight, 1964). The labelled cells were washed once in medium " 199 " (Morgan, Morten and Parker, 1950) and resuspended in fresh " 199 " for intravenous injection into the recipient rats.

Cell suspensions.—Lymphocytes were obtained from the thoracic duct of rats by the method of Bollman, Cain and Grindlay (1948). Lymph was collected at room temperature into sterile flasks containing 5 ml. of Krebs-Ringer solution, 100 units of heparin and 0·5 mg. of streptomycin. Individual collections were made for periods up to 12 hr. The lymph was centrifuged for 10 min. at 100 *g.* and the pellet was resuspended in " 199 " for intravenous infusion into the recipients. In other experiments the lymphocytes were labelled *in vitro* with tritiated adenosine before their injection, as described above.

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Suspensions of cells were prepared from lymph nodes, thymus, spleen and femoral bone marrow by teasing the tissues in " 199 ", filtering through gauze and centrifuging for 10 min. at 100 *g*. The pellet of cells was resuspended in 1–2 ml. of " 199 " for intravenous injection into the recipient rats.

X-irradiation.—Unanaesthetized rats were X-irradiated in aluminium cylinders and received a mid-phantom dose for muscle of either 400 or 750 rads (Corp, 1957). The irradiations were performed at the Radiobiological Research Unit, Harwell.

RESULTS

Removal of Possible Sources of Macrophages

Splenectomy

Four rats were splenectomized 2 days after a subcutaneous dose of neoarsphenamine (2 mg. per 100 g. body weight) which was given as prophylaxis against *Bartonella muris* infection (Mayer, Borchardt and Kikuth, 1927). Six days after splenectomy coverslips were inserted under the skin of each rat and removed after 24 hr. In one rat, a single dose of tritiated thymidine was given 12 hr. before the insertion of the coverslips.

Abundant macrophages were present on all the coverslips and a normal proportion of them became labelled after an injection of tritiated thymidine : 39·4 and 44·0 per cent of the macrophages respectively were labelled on the 2 coverslips which were removed from the injected rat. Thus, if the spleen is a source of macrophages it is not the only source.

Thymectomy

Coverslips were inserted subcutaneously into one rat on the 8th day and into another on the 12th day after complete thymectomy and removed after 24 hr. ; the first of these two rats received a single injection of tritiated thymidine 24 hr. before the insertion of the coverslips. Neither the abundance of macrophages in the exudates nor the proportion of macrophages which became labelled were reduced by prior thymectomy : 48·4 and 35·5 per cent of macrophages respectively were labelled on the 2 coverslips from the rat which received tritiated thymidine.

Lymphocyte-depletion

If small lymphocytes are the precursors of macrophages (Rebuck and Crowley, 1955) then it should be possible to reduce the number of macrophages which can be mobilized into an inflammatory exudate by first depleting an animal of its lymphocytes.

Four rats were depleted of lymphocytes by draining lymph and cells from a thoracic duct fistula for 5 days. This procedure reduces considerably the number of small lymphocytes in lymphoid tissue and also leads to a fall of about 90 per cent in the concentration of small lymphocytes in the blood (McGregor and Gowans, 1963). The lymphocyte-depleted rats were temporarily removed from their restraining cages for the insertion of subcutaneous coverslips. These were left in place for 24 hr. while lymph continued to drain from the fistulae. In one rat a single dose of tritiated thymidine was given 12 hr. before the insertion of the coverslips.

The coverslips from all the animals showed cellular exudates which were quantitatively and qualitatively indistinguishable from those obtained from normal

rats. A large range of mononuclear cells from " small round cells " to large macrophages was represented. The percentages of labelled macrophages on the two coverslips from the rat which received tritiated thymidine were within the normal range : 44·5 and 37·5 per cent respectively (Fig. 1).

Total body X-irradiation

A series of experiments was performed to determine whether the damage which can be inflicted on lymphoid tissue and bone marrow by a dose of X-irradiation would reduce or abolish the macrophage response on glass coverslips. The rats were given either a sublethal dose of 400 rads, or 750 rads which is in the lethal range.

X-irradiation with 400 *rads.*—Coverslips were placed on or under the skin of 3 rats 24 hr. after irradiation and removed 24 hr. later. One further rat was given tritiated thymidine 24 hr. after irradiation and had coverslips applied 12 hr. later ; these were again removed after 24 hr.

All the coverslips showed abundant macrophages and a normal proportion of them was labelled in the rat receiving tritiated thymidine : 34·2 per cent were labelled on a skin window and 33·8 and 27·6 per cent respectively on 2 subcutaneous coverslips. A blood sample which was taken from this rat while the coverslips were still in place contained 330 lymphocytes per cu. mm., less than 10 per cent of the normal value.

X-irradiation with 750 *rads.*—Ten rats were given 750 rads and then treated in the same way as the group which received 400 rads ; 7 of the rats were given an injection of tritiated thymidine.

There were few macrophages on any of the coverslips but among these many possessed a highly vacuolated cytoplasm which contained nuclear debris or fragments of erythrocytes. Table I shows that, with one exception, very few labelled macrophages appeared after the injection of tritiated thymidine. The explanation for the behaviour of the exceptional animal is not known.

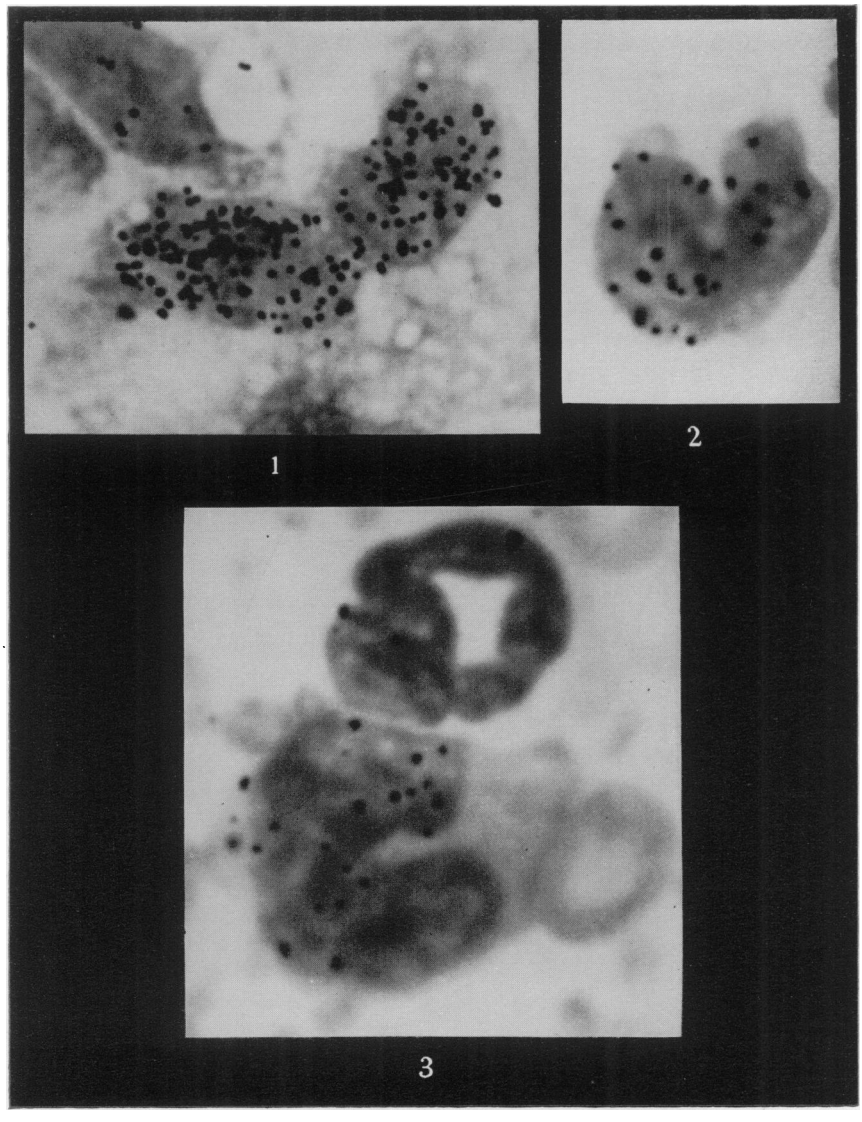
X-irradiation with 750 *rads together with marrow shielding.*—The failure of lymphocyte-depletion and of 400 rads of X-irradiation to influence the emergence of labelled macrophages argues against lymphoid tissue as being a major source of macrophage precursors. On the other hand, the suppressive effect of 750 rads suggests that they may normally reside in bone marrow. An attempt was therefore made to restore the macrophage response to normal by shielding the bone marrow in rats which received 750 rads.

Two unanaesthetized rats received 750 rads while one hind limb was shielded with 3 mm. of lead distal to the popliteal fossa. Each rat was given a single dose

EXPLANATION OF PLATE

FIG. 1.—Autoradiograph of macrophages on a skin window which had been in place for 24 hr. in a rat depleted of lymphocytes by chronic drainage from the thoracic duct. A single injection of tritiated thymidine, which had been given 12 hr. before the coverslip was applied, labelled about 40 per cent of the macrophages in this preparation. (Exposure, 30 days. May-Grünwald Giemsa. ×2250.)

FIGS. 2 and 3.—Labelled monocytes in leucocyte concentrates from a rat given an intravenous injection of syngeneic bone marrow cells 24 hr. previously. The marrow was taken from the donor 5 hr. after a single injection of tritiated thymidine. (Exposure, 14 days. May-Grünwald Giemsa. ×3000.)



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of tritiated thymidine 12 hr. after X-irradiation. Skin windows and subcutaneous coverslips were applied 24 hr. after the injection of tritiated thymidine and were left in place for 24 hr. Table I shows that shielding of the tibial marrow during irradiation restored the proportion of labelled macrophages to normal in the two animals.

TABLE I.—*The Effect of Marrow Shielding on the Percentage of Labelled Macrophages on Coverslips Applied* 12 *hr. After a Single Dose of Tritiated Thymidine and* 36 *hr. After* 750 *rads X-irradiation.*

No. rats	Treatment	per cent macrophages labelled on individual coverslips†	Total macrophages counted per rat
7	750 rads	0, 0	577
		0·8,* 0·5	312
		1·4, 0	171
		0·5, 0	400
		21·1,* 23·4*	138
		0	100
		6·0	100
2	750 rads + marrow shielding	38·5,* 30·7	1775
		49·5,* 27·5	400

*Skin windows; other preparations were subcutaneous coverslips.
†Pairs of values for a single rat are counts on each of two coverslips from the same animal.

Transfusion of Labelled Cell Suspensions

Further attempts to locate the precursors of macrophages were made by applying skin windows and subcutaneous coverslips to rats which had been transfused with suspensions of radioactively labelled cells prepared from the lymphoid tissue and bone marrow of syngeneic donors. In addition, the idea that lymphocytes might be the circulating antecedents of macrophages was tested by transfusing radioactively-labelled cells obtained from the thoracic duct.

Thoracic duct lymphocytes

Inoculum labelled in vivo *with tritiated thymidine.*—A single injection of tritiated thymidine was given to each of 4 donor rats about 8 hr. after cannulation of the thoracic duct. A 12 hr. collection of lymph from one donor was begun immediately after the injection of tritiated thymidine and yielded 800 million cells. These were transfused into the femoral vein of a recipient rat over a period of 4 hr. by means of a roller pump. Two skin windows had been applied to the recipient immediately before the transfusion: one was removed after 24 hr. and replaced by another; the two which then remained were removed after a further 24 hr.

Lymph from the other 3 donor rats was pooled and the start of the collection was delayed until 12 hr. after the injection of tritiated thymidine in order to correspond to the time at which labelled macrophage precursors are known to emerge into the blood (Volkman and Gowans, 1964). Cells from the pooled collections were injected with a syringe in 2 doses, 12 hr. apart, into each of 2 recipients; one received a total of 290 million and the other 800 million cells.

Subcutaneous coverslips were inserted immediately before the first injection and removed 24 hr. after the second.

No labelled macrophages appeared on the coverslips of the recipients. Each recipient received an inoculum in which 6–7 per cent of the cells were labelled and, of these about 83–86 per cent were large lymphocytes.

Inoculum labelled in vitro *with tritiated adenosine.*—The previous experiment provided no evidence that the large lymphocytes which enter the blood with the thoracic duct lymph can develop into macrophages. In order to test whether small lymphocytes have this potentiality cells from the thoracic duct of donor rats were first incubated *in vitro* with tritiated adenosine. This labelled 66 and 85 per cent respectively of all the small lymphocytes in the 2 inocula which were prepared for the present study; in addition virtually all the large lymphocytes, which made up 5 per cent of the total inoculum, were labelled.

Skin windows were applied to 2 rats immediately before the intravenous injection of labelled lymphocytes and were removed from one rat after 24 hr. and from the other after 36 hr.; one rat received 282 million and the other 564 million labelled small lymphocytes. No labelled macrophages appeared on the coverslips in either rat. The fate of small lymphocytes which have been labelled *in vitro* with tritiated adenosine can be followed for several days after their transfusion into syngeneic recipients (Gowans and Knight, 1964) and a transformation of labelled small lymphocytes into large pyroninophilic cells has been identified during a graft-versus-host reaction (Gowans, 1962). There are thus grounds for supposing that if any small lymphocytes from the thoracic duct had transformed into macrophages, then some of them would have been identified.

Cells from lymph nodes, thymus, spleen and bone marrow

Skin windows or subcutaneous coverslips were applied to 8 rats immediately before they were given intravenous injections of cells prepared from either the lymph nodes, thymus, spleen or bone marrow of syngeneic donor animals which had received injections of tritiated thymidine. Suspensions of cells were prepared from one donor 5 hr. after the last of 4 12-hourly injections of tritiated thymidine, and from 6 donors 5 hr. after a single injection. The coverslips were removed from the recipients after 24 hr., except in a single experiment (recipient 8, Table II) in which the first coverslip was removed at 21 hr. and replaced by a second which was removed 33 hr. later.

Table II shows that radioactively labelled bone marrow consistently provided a source of labelled macrophages on subcutaneous coverslips. The number of cells in an inoculum of bone marrow which could conceivably give rise to labelled macrophages is considerably smaller than the total number of cells injected. In Table II estimates are recorded of the number of " potentially effective " marrow cells infused into the recipients. These estimates exclude cells which are recognizably committed to another line of development: for example, granulocytes and erythrocyte precursors. It can be seen that an injection of about 60 million labelled " potentially effective " marrow cells yielded up to 7·9 per cent of labelled macrophages on subcutaneous coverslips. The spleen was also a source of macrophage precursors, but none was detected in the thymus or lymph nodes.

The evidence that bone marrow contains macrophage precursors was strengthened by the observation that labelled monocytes were found in the blood

TABLE II.—*Percentage of Labelled Macrophages on Skin Windows (SW) and on Subcutaneous Coverslips (SC) in Rats after the Intravenous Infusion of Tritium-labelled Lymphoid and Bone Marrow Cells*

Donor rats			Recipient rats				
						Macrophages on coverslips	
Origin of cell suspension	No. injections of tritiated thymidine	Rat no.	Total no. cells infused ($\times 10^6$)	No. labelled cells infused ($\times 10^6$)	Type of preparation	No. counted	Per cent labelled
Lymph node	1	1	200	20	SW	500	0
					SC	500	0
Thymus	4	2	570	200	SC	1000	0
	1	3	470	75	SC	1000	0
Spleen	1	4	290	14·5	SC	1000	0·2
	1	5	600	44	† SC	500	1·0
					SC	500	0·6
Bone* Marrow	4	6	132(27)	(19·7)	† SC	1000	0·4
					SC	1000	2·3
	1	7	270(43)	(25·8)	† SW	1000	1·5
					SC	1000	1·4
	1	8	464(101)	(60)	† SC	1000	7·9
					SC	1000	5·0
					‡ SC	500	0·6
					SC	1000	2·8

*Values in parenthesis are estimates of the number of "potentially effective" marrow cells infused (see text).

†Pairs of coverslips on the same animal.

‡First coverslip in place 0–21 hr. after infusion ; second coverslip, 21–54 hr. (see text).

of 2 of the recipients of labelled marrow cells : 15 out of 1000 monocytes were labelled in blood films from one rat, and 4 out of 200 in those from another (Fig. 2 and 3).

Small numbers of labelled cells, indistinguishable morphologically from small lymphocytes, were seen on the coverslips from animals receiving bone marrow, but never in recipients of the other tissues. Whether they represented a stage in the development of macrophages or were merely artefacts of the method of preparation is not known.

DISCUSSION

In the preceding paper it was argued that the ability of a single injection of tritiated thymidine to label up to 60 per cent of the macrophages on skin windows in the rat was incompatible with an origin of macrophages from small lymphocytes (Volkman and Gowans, 1964). This conclusion is reinforced by the findings in the present study that neither the emigration nor the labelling of macrophages was affected in rats depleted of lymphocytes by 400 rads of X-irradiation or by chronic drainage from a thoracic duct fistula. Further, the transfusion of radioactively-labelled large and small lymphocytes from the thoracic duct, and of suspensions of cells prepared from lymph nodes and thymus did not lead to the appearance of labelled macrophages on coverslips borne by syngeneic recipients. The failure to observe a transformation of lymphocytes into macrophages has also been reported by Beckfield and Hirata (1961) after the injection of thoracic duct cells labelled with acridine orange into granuloma pouches of rats, and by Ebert, Sanders and

Florey (1940) who watched individual lymphocytes in the tissues of rabbit ear chambers.

Two experiments strongly suggest that the precursors of the exudate macrophages proliferate in the bone marrow. First, it was found that although the migration of macrophages and their labelling after an injection of tritiated thymidine were suppressed by 750 rads of X-irradiation, both could be restored to normal by shielding the bone marrow during irradiation ; and second, that labelled cell suspensions prepared from the bone marrow and injected intravenously into syngeneic recipients gave rise to labelled macrophages on the coverslips. The work of Balner (1963) supports the idea that macrophages originate from bone marrow. He found that the free peritoneal macrophages in mouse radiation chimeras were all of donor-type 6 weeks after receiving an injection of allogeneic bone marrow. A similar finding was reported by Goodman (1964).

Although the evidence for the origin of exudate macrophages from bone marrow is very strong it cannot exclude the possibility that small numbers of macrophage precursors may proliferate in lymphoid tissue or that an occasional precursor may gain entrance into the lymph after having been formed elsewhere. Some force is lent to this possibility by the finding of Schooley and Berman (1960) that " monocytoid " cells arose in diffusion chambers from some of the large or medium lymphocytes in thoracic duct lymph. However, the labelling characteristics of exudate macrophages after a single injection of tritiated thymidine, the results of the transfusion experiments, and the unperturbed emergence and labelling of macrophages in rats with open thoracic duct fistulae suggest that if macrophages can arise in lymphoid tissue then the contribution of this source to the macrophages on skin windows is negligible.

It is possible that the labelled macrophages which appeared on coverslips after the injection of labelled spleen cells also arose from myeloid elements. The alternative possibility is that they arose from the cells of the reticuloendothelial system. In this connection it was noted that after 4 12-hourly injections of tritiated thymidine 1–4 per cent of the Kupffer cells in sections of rat liver were labelled, a figure which is in agreement with that found in mice by Kelly, Brown and Dobson (1962) and by Mims (personal communication). Thus, the large, slowly proliferating mass of sinusoidal histiocytes in the liver could provide a potential source of newly-formed phagocytes. On the other hand, bone marrow transfusions provided relatively few potential precursors but gave rise to significant numbers of labelled macrophages on the coverslips. This finding, together with the observation that a large proportion of the coverslip macrophages were labelled after a single dose of tritiated thymidine (Volkman and Gowans, 1965), suggest that the labelled macrophages originated from a small but rapidly proliferating pool of precursors.

The experiments described in the accompanying paper showed that the precursors of macrophages released their progeny into the blood where they underwent very little further cell-division. The morphological identity of these circulating cells is not known but one obvious candidate is the blood monocyte. Thus, it has been noted that the pattern of labelling among exudate macrophages after a single injection of tritiated thymidine is very similar to that of the blood monocytes (Volkman and Gowans, 1965), and in the present study blood monocytes were shown to arise from precursors in bone marrow. Witts and Webb (1927), while studying an experimental infection with *Listerella monocytogenes* in rabbits noted

that the blood monocytosis was associated with a hyperplasia of monocytoid forms in the bone marrow and spleen ; and there is no doubt that blood monocytes can emigrate into the tissues and become macrophages (Ebert and Florey, 1939). However, it is tempting to suggest that at least some of the cells which originate in bone marrow and are destined to appear in the tissues as macrophages may, during their circulatory and migratory phases, resemble small lymphocytes rather than monocytes. The presence of labelled " small round cells " in exudates obtained from lymphocyte-depleted animals and from the recipients of labelled bone marrow (but not from the recipients of labelled cells from the lymph nodes, thymus or thoracic duct lymph) supports this idea and would also explain the confusion that has resulted from attempts to analyse morphologically the succession of mononuclear cells which appear on skin windows (Rebuck and Crowley, 1955). Such " small round cells " would be " small lymphocytes " only in a narrow morphological sense for they would have to be distinguished both in origin and in function from the small lymphocytes in lymph and in lymphoid tissue. It has recently been reported that cells resembling small lymphocytes are produced within the bone marrow (Osmond and Everett, 1964) and may function as haemopoietic stem cells (Schooley and Giger, 1962 ; Cudkowicz, Upton, Shearer and Hughes, 1964). These important findings again point to the existence of different classes of " small round cells " in the animal and emphasize the inadequacy of conventional morphological criteria for distinguishing between them.

Forbes and Mackaness (1963) have shown that when sensitized mice are challenged with antigen the peritoneal macrophages divide at a rapid rate and incorporate tritiated thymidine *in vitro*. They also noted the presence of small round cells in the peritoneal fluid and intermediate forms between these and the macrophages. The findings of Forbes and Mackaness could be reconciled with the present study if it is postulated that a dividing precursor of macrophages is mobilized from the marrow into the blood as a result of the antigenic challenge. However, it remains to be determined whether the macrophages in acute inflammation induced by mild trauma to the skin and those evoked during immunological responses in sensitized animals are derived from identical precursors.

SUMMARY

" Skin windows " and subcutaneous coverslips were applied to rats in a study designed to identify the tissues in which the precursors of macrophages proliferate.

Lymphocyte-depletion by either chronic drainage from the thoracic duct or 400 rads of X-irradiation failed to suppress the emigration of macrophages or to reduce the proportion of them which became labelled after an injection of tritiated thymidine.

X-irradiation with 750 rads suppressed the emigration and the labelling of the exudate macrophages. Both were restored to normal when the tibial marrow was shielded during irradiation.

Radioactively-labelled cell suspensions obtained from thoracic duct lymph, lymph nodes, thymus, spleen and bone marrow were transfused into syngeneic recipients. The emigration of labelled macrophages on to coverslips could be demonstrated only in recipients of labelled bone marrow and spleen cells. Labelled monocytes were found in the blood of rats which had received injections of labelled bone marrow.

It was concluded that in the rat, bone marrow, and to a lesser extent spleen, are major sources of the macrophages which emigrate into foci of acute, non-bacterial inflammation.

We should like to thank Mrs. Janet Crawford, Miss Judith Stow and Mr. J. H. D. Kent for valuable technical assistance, and Mr. S. A. Buckingham for the photographs. We are extremely grateful to the Director, Radiobiological Research Unit, Harwell, for providing the facilities for X-irradiating animals. These studies were performed during the period when one of us (A.V.) was Scholar of the American Cancer Society.

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