THE ORIGIN AND TURNOVER OF MONONUCLEAR CELLS IN PERITONEAL EXUDATES IN RATS*

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In a previous report the population dynamics of emigrative macrophages in rats was described (1). The macrophages were obtained from foci of acute nonbacterial inflammation provoked on abraded skin surfaces or in subcutaneous tissues at varying intervals with respect to the time of a single injection of tritiated thymidine (TdR-H^3). The results indicated that the macrophages which adhered to glass cover slips in contact with the sites of inflammation were the progeny of rapidly and continuously dividing precursors. Similar conclusions have been reached by others in a variety of experimental situations (2–5). It was also shown that the macrophages emigrating to cutaneous and subcutaneous foci of acute nonbacterial inflammation originated predominantly from bone marrow (6). The origin of peritoneal macrophages from donors' bone marrow has been demonstrated by Balner (7) and Goodman (8) in lethally irradiated mice. However, the traditional view, among whose proponents have been Cappell (9), and more recently Felix and Dalton (10), is that the majority of peritoneal mononuclear cells arise from local elements.

The purpose of the present study has been to determine whether the macrophages of experimentally induced peritoneal exudates have isotopic labeling features which suggest their derivation from the same precursors as those macrophages previously characterized (1, 6). Evidence is presented which supports this idea. Lymphocytelike cells in the exudates were also shown to be members of a rapidly proliferating population with precursors in bone marrow.

Materials and Methods

Animals.—Randomly-bred Columbia-Sherman rats of both sexes weighing between 100 to 200 g were used in most of the experiments. Only highly inbred BN rats (Microbiological Associates Inc., Walkersville, Maryland.) were used as partners in parabiosis experiments and as donors and recipients of cell transfusions. All operative procedures were carried out with the rats under ether anesthesia.

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Tritiated Thymidine Labeling.—Rats were injected in a tail vein with a single dose of 1 μc of TdR-H^3 per gram of body weight (specific activity, 3.0 c/mM; Schwarz BioResearch Inc., Orangeburg, New York).

Peritoneal Exudates.—Cellular exudation was induced at varying intervals with respect to the time of TdR-H^3 injection by the intraperitoneal (i.p.) instillation of 5 ml of 1% rabbit liver glycogen (Mann Research Laboratories, Inc., New York). 24 hr later each rat was bled from the heart and 20 ml of Hanks' balanced salt solution were injected i.p. Ten to 12 ml aliquots of fluid were aspirated after briefly massaging the rat's abdomen. The cells were counted in a conventional hemacytometer. After the washings were centrifuged at 500 g for 10 min the pellets were resuspended in a drop of bovine serum and smeared on glass slides. The smears were air-dried, fixed in methyl alcohol, and extracted in 5% trichloroacetic acid at 4°C before the preparation of radioautographs. Representative smears were stained with May-Gruenwald Giemsa and the percentages of mononuclear cells were determined by counting 200 nucleated cells per rat. In the same smears, 200 mononuclear cells from each rat were counted to determine the proportion which morphologically resembled small lymphocytes.

Parabiosis.—A modification of the Sauerbruch-Heyde technique (11) was used to join pairs of highly inbred rats. The peritoneal cavities were not in communication. 1 to 2 months after union the common circulation was occluded by the application of atraumatic intestinal clamps while one rat (donor) was injected with a single intravenous dose of TdR-H^3. 1 hr later the clamps were removed. In this period most of the radioactivity as thymidine is known to be dissipated. Absence of label from rapidly proliferating locally formed cells in the noninjected (recipient) rat's intestinal epithelium indicated in each case that no leakage of TdR-H^3 had occurred. Therefore, when labeled cells were found in the recipient, it was concluded that they had been formed in the donor and had arrived via the circulation.

Cell Transfusions.—Bone marrow was flushed from the tibias of donor rats with cold (4°C) tissue culture medium 199 (Microbiological Associates, Inc., Bethesda Maryland) 3 hr after the rats were labeled with a single injection of TdR-H^3. Thoracic duct lymphocytes were collected from other labeled rats in the manner previously described (6). All cell suspensions were filtered through surgical gauze and then centrifuged at 500 g for 10 min and resuspended in 1 to 2 ml of fresh No. 199 for intravenous injection into the recipient rats. In additional experiments thoracic duct lymphocytes were collected from unlabeled rats and were incubated in vitro in No. 199 for 1 hr at 37°C with tritiated adenosine at a concentration of 10 μc/ml (specific activity 1 c/mM; Schwarz BioResearch, Inc.). This procedure labeled a high proportion of small lymphocytes and virtually all of the medium and large lymphocytes. The labeled cells were washed once and resuspended in fresh No. 199 for injection into the recipient rats. Smears for radioautography were prepared from small residua of the suspensions and processed in the same manner as peritoneal exudate cells.

Radioautography.—Unstained smears of peritoneal exudate cells, bone marrow, and thoracic duct lymphocytes were dipped into Eastman Kodak liquid emulsion NTB-3 which had been diluted 3:1 with distilled water. The coated slides were thoroughly dried in a current of air and exposed in light tight boxes at 4°C for 2 wk. They were developed in Eastman Kodak developer No. D-11 and subsequently stained with May-Gruenwald Giemsa.

RESULTS

Cells of the Exudates.—The usual variety of granulocytes and mononuclear cells was observed and their relative numbers (Table I) found to be in agreement with the figures given by Brumfitt and Glynn (12). The vast majority of the mononuclear cells were monocytelike cells or macrophages which will be referred to as macrophages throughout the paper. These cells were large, 15 to

50 μ, in diameter, with pale nuclei having a relatively fine chromatin meshwork. The nuclei frequently appeared indented or partially folded. Their cytoplasm was relatively abundant, pale, and often vacuolated. Fragments of phagocytized debris were often found within the cytoplasm. A juxtanuclear clear space was commonly seen in these cells. There were in addition a smaller percentage of cells which were morphologically indistinguishable from small lymphocytes of blood or thoracic duct lymph. The studies were restricted to these readily classifiable cell groups without any attempts to evaluate possible transitional forms.

Table I also shows the proportion of lymphocytelike cells which were present. Cells thus categorized were between 7 to 9 μ in diameter with dense pachychromatic nuclei and scant cytoplasm.

TABLE I

Cell Contents in Washings of Peritoneal Exudates Harvested 24 Hr after Glycogen Injection

	Range	Mean ± SE
Cells/ml × 10^6	1.6-9.2	3.9 ± 0.29
Mononuclear cells, %	35.0-74.6	57.6 ± 1.38
Small lymphocytes, %*	3.0-25	14.1 ± 0.86

* Calculated with respect to 200 mononuclear cells/rat.

Radioautographic Labeling.—

(*a*) *Direct labeling:* 24 hr after glycogen instillation 3 rats were each given a single dose of TdR-H^3 i.p. and the exudates were harvested 1 hr later to minimize contamination due to possible entry of cells labeled at other sites after diffusion of TdR-H^3 from the peritoneal cavity. Exudates were also collected from three nonlabeled rats and incubated in vitro for 1 hr at 37°C in medium 199 containing 1 μc of TdR-H^3 per milliliter.

Table II shows close agreement between the proportions of mononuclear cells which can be labeled directly in vivo and in vitro. It can be concluded that under the conditions of the experiments only a relatively small proportion of the mononuclear cells are capable of incorporation of TdR-H^3. This is compatible with the activity of macrophages on cover slips (1) and the reports of others which indicate little or no proliferative activity among peritoneal macrophages in simple inflammation (13, 14). However, under special conditions not operative in the present experiments, an increased frequency of mitosis has been observed (15).

(*b*) *Labeling of macrophages:* Table III shows that there is a distinct pattern of variation of the proportion of labeled macrophages with respect to the time of TdR-H^3 injection. This is shown graphically in Text-fig. 1. A single pulse of

TdR-H^3 in which the radioactivity as thymidine is only briefly available can result in the labeling over 40% of the macrophages. This peak appears to be reached when TdR-H^3 is given 24 to 48 hr before the induction of inflammation. In view of the fact that the mononuclear exudate cells display a limited ability to incorporate TdR-H^3 (section *a*), it can be concluded that the abundance of labeled cells is a reflection of the rapid proliferation of macrophage precursors. If the macrophages are rapidly formed it would be anticipated that the labeled antecedent population would quickly be dissipated either by rapid dilution of label due to precursor division or the death of cells at a rate matched to their rate of production, if a steady state mechanism were operative. As expected a reduced proportion of labeled macrophages was obtained when TdR-H^3 was

TABLE II

Direct Labeling of Mononuclear Cells with TdR-H^3 in 24 Hr Peritoneal Exudates in Rats

	Rats	Labeled
		%
In Vivo	1*	0.8
	2	0.6
	3	1.2
In Vitro	4	0.4
	5	1.6
	6	1.2

Percentages derived from counts of 500 mononuclear cells per rat. Exposure of cells to TdR-H^3 in both groups was 1 hr (see text).

* Number designations are arbitrary and do not indicate identity with rats similarly numbered in other tables.

given 96 hr before inflammation was induced. Since most of the inflammations were induced after radioactivity in the form of thymidine was no longer available, the proliferation of macrophage precursors must be continuous. The findings correspond in general with the data obtained for the labeling of macrophages in skin and subcutaneous exudates (1) (see Fig. 1).

(*c*) *Labeling of lymphocytes:* At certain intervals after a single dose of TdR-H^3 relatively large proportions of the lymphocytes in peritoneal exudates become labeled (Table III). This relationship is shown graphically in Text-fig. 2. A maximum of about 40% was reached when TdR-H^3 was given 6 hr before the induction of inflammation but the percentage labeled was very low when the interval between TdR-H^3 administration and glycogen injection was increased to 96 hr. When the data for the labeling of each cell type are compared it is apparent that the generalizations regarding the macrophages are also applicable to the small lymphocytes in the exudates: the cells are derived from rapidly

proliferating precursors whose division is continuous. The antecedents of the lymphocytes in the exudates apparently are also short lived. These findings contrast sharply with the reported values which indicate the slow turnover and longevity of the majority of small lymphocytes in blood and thoracic duct lymph (16–18) (see Fig. 2).

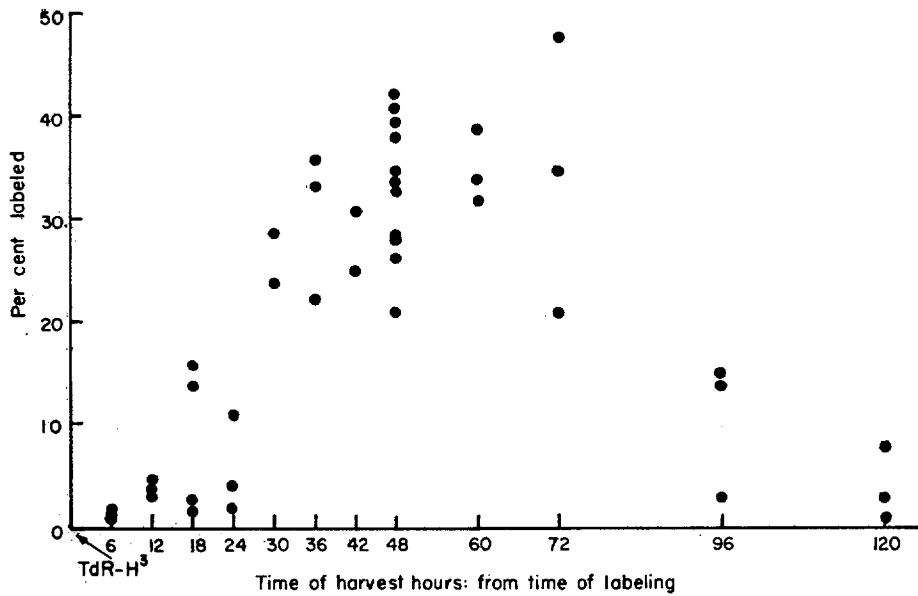
TABLE III

Percentage of Labeled Macrophages and Small Lymphocytes in Glycogen-induced Peritoneal Exudates of 24 Hr Duration Harvested at Varying Intervals with Respect to the Time of Pulse Labeling with TdR-H³

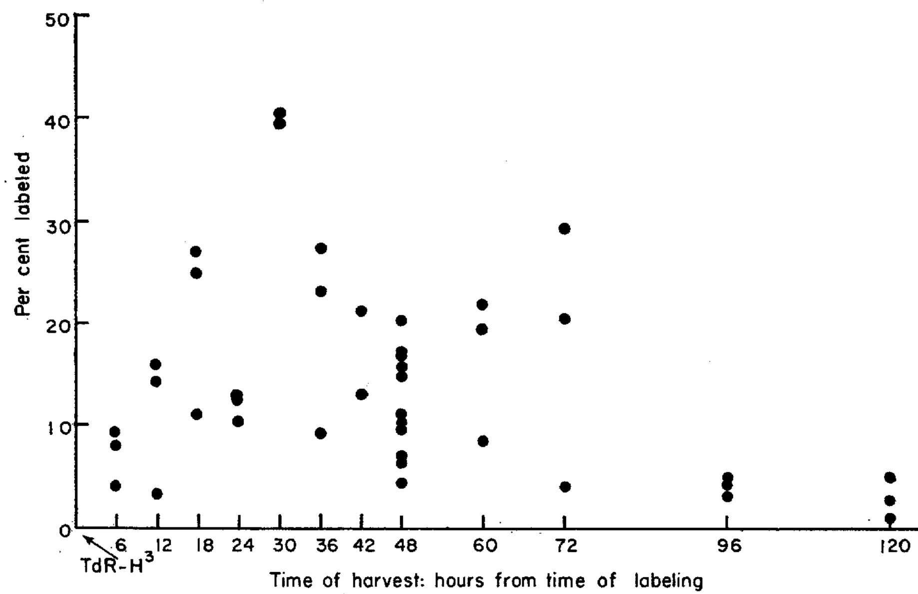
Rat No.	Time of harvest (after TdR-H³)	Macrophages labeled	Lymphocytes labeled	Algebraic difference	Rat No.	Time of harvest	Macrophages labeled	Lymphocytes labeled	Algebraic difference
	hr	%	%			*hr*	%	%	
1	6	1.5	4.0	−2.5	22	48	28.5	11.5	+17.0
2	6	1.0	8.0	−7.0	23	48	21.5	7.0	+14.5
3	6	1.5	9.5	−8.0	24	48	42.5	10.5	+32.0
4	12	2.4	3.5	−1.1	25	48	26.5	15.0	+11.5
5	12	3.0	16.0	−13.0	26	48	38.0	17.5	+20.5
6	12	2.5	14.0	−11.5	27	48	28.6	17.5	+11.1
7	18	1.6	11.0	−9.4	28	48	39.5	20.0	+19.5
8	18	14.4	25.0	−10.6	29	48	21.5	4.5	+17.0
9	18	16.0	27.0	−11.0	30	48	34.0	16.0	+18.0
10	24	11.2	13.0	−1.8	31	60	39.0	22.0	+17.0
11	24	4.0	12.5	−8.5	32	60	34.0	19.5	+14.5
12	24	2.0	10.5	−8.5	33	60	32.0	8.5	+23.5
13	30	29.0	39.5	−10.5	34	72	48.0	20.5	+27.5
14	30	24.0	40.5	−16.5	35	72	21.0	4.0	+17.0
15	36	33.5	25.5	+8.5	36	72	35.0	27.5	+7.5
16	36	22.5	9.3	+13.2	37	96	3.2	3.5	−0.3
17	36	36.0	27.5	+8.5	38	96	15.5	5.5	+10.0
18	42	25.3	21.5	+3.8	39	96	14.0	4.5	+9.5
19	42	31.0	13.0	+18.0	40	120	3.0	1.0	+2.0
20	48	33.5	9.4	+24.1	41	120	1.0	3.0	−2.0
21	48	35.5	6.5	+29.0	42	120	8.0	5.5	+2.5

Percentages were derived from counts of 200 to 500 cells of the respective cell types per rat.

Certain discrepancies are apparent when Text-fig. 1 and Text-fig. 2 are compared. Both the rise and fall in the percentage of labeled cells occur at relatively earlier intervals among the lymphocytes than among the macrophages. This results in a numerical difference between the percentages of each of the two cell types labeled in an individual animal (Table III). The number of rats sampled at most of the intervals are too few to determine whether the differences at each interval are due to chance. However, at the 48 hr harvest



TEXT-FIG. 1



TEXT-FIG. 2

TEXT-FIGS. 1 and 2. Percentages of labeled peritoneal macrophages (1) and lymphocytes (2) in rats sacrificed 24 hr after i.p. instillation of glycogen. Inflammation was induced at varying intervals with respect to the time of a single injection of TdR-H^3. Each point in the chart was obtained from one animal and is recorded at the time of harvest. For the percentage labeling of the pairs of cell types in each rat see Table III.

time there were sufficient samples to permit evaluation with a conventional t test corrected for correlated means (19): $P < 0.001$. In addition, if the algebraic differences between the two cell types recorded in Table III were due to chance the order of their signs should be random and their sum 0. On the contrary, the first 14 differences are negative and all but two of the remainder positive. The probability of 14/15 samples having the same sign obviously must be exceedingly low and weighs heavily against chance. Furthermore, when the paired differences in the entire series are analyzed with Wilcoxon's matched pairs–signed ranks test (20), $P < 0.01$.

TABLE IV

Labeling of Mononuclear Cells in Blood and Peritoneal Exudates in Parabiotic Rats 48 Hr after a Single Injection of TdR-H^3

Rat pairs	Peripheral blood				Peritoneal exudates (24 hr)			
	Small lymphocytes		Monocytes		Small lymphocytes		Macrophages	
	Counted	%	Counted	%	Counted	%	Counted	%
Donor	1/500	0.2	40/200	20.0	48/200	24.0	80/200	40.0
Recipient	2/500	0.4	29/200	14.5	25/200	12.5	25/200	12.5
Donor	4/500	0.8	87/200	43.5	24/200	12.0	104/200	52.0
Recipient	1/500	0.2	20/200	10.0	15/200	7.5	44/200	22.0
Donor	4/339	1.2	32/105	30.5	24/200	12.0	180/500	36.0
Recipient	3/381	0.8	20/107	18.7	18/200	9.0	100/500	20.0

The two cell types therefore represent two distinct populations in the statistical sense. This does not necessarily mean that the two represent ontogenetically distinct populations. Alternatively, the difference may be due to the time required for differentiation or maturation of the smaller cells to the larger. This speculation is tempting in view of the overall similarity of the respective labeling patterns.

Parabiosis.—In Table IV the labeling of small lymphocytes and monocytes in blood is compared with the labeling of small lymphocytes and macrophages in peritoneal exudates. Up to 22% of the macrophages were labeled in peritoneal exudates in the recipient rats. As previously explained, these cells must have travelled from the donors to the recipients via the common circulation since there was no evidence of TdR-H^3 leakage in the control tissues examined. The figures for the proportion of mononuclear exudate cells which can emigrate from the blood should be regarded as minimum values since the precursors of the exudate cells were exposed to only a single pulse of TdR-H^3. Other impediments

to circulatory exchange in the parabiosis system may also be operative. A justifiable extrapolation from the data suggests therefore, that most of the macrophages are hematogenous, as would be expected from the findings relating to macrophages in skin (1).

TABLE V

Percentage of Labeled Macrophages in 24 Hr Peritoneal Exudates 48 Hr after the Transfusion of Isotopically Labeled Bone Marrow Cells

Total No. cells transfused ($\times 10^6$)	No. Labeled cells transfused* ($\times 10^6$)	Recipient macrophages Labeled
		%
260	15.6	0.7
390	23.4	1.4 ‡
1900	72	1.3 ‡

1000 macrophages were counted in the exudate of each recipient.

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

‡ In addition: recipient 2, 5% of 200 small lymphocytes labeled; and recipient 3, 0.6% of 500 small lymphocytes labeled.

TABLE VI

Percentage of Labeled Macrophages in 24 Hr Peritoneal Exudates 48 Hr after the Transfusion of Isotopically Labeled Thoracic Duct Lymphocytes

Total No. cells transfused ($\times 10^6$)	No. Labeled cells transfused ($\times 10^6$)	Recipient macrophages	
		No. Counted	Labeled
			%
500	33 *	500	0
528	8 *	1000	0.1
700	630 ‡	1000	0
1500	1190 ‡	1500	<0.1 (1 cell)
470	340 ‡	1000	0

* Labeled in vivo with a single injection of $TdR\text{-}H^3$ over 90% of large and medium lymphocytes and about 1% of small lymphocytes labeled.

‡ Labeled in vitro with $Adenosine\text{-}H^3$: 70 to 85% of small lymphocytes and virtually all large and medium lymphocytes labeled.

A relatively high proportion of labeled lymphocytes was also recovered from the exudates of recipient rats. Although these percentages are lower than those observed among macrophages they are consistent with the yields in individual rats at the 48 hr harvest (Table III). Extrapolating again: a high proportion of these exudate lymphocytes with an apparently fast turnover must be derived

from cells in the blood. Table IV also shows that there is some correspondence in labeling between the proportions of labeled small lymphocytes in the blood and the lymphocytelike cells in the exudates. Since a considerable proportion of the latter cells emigrate from the blood and are morphologically indistinguishable from other small lymphocytes, it must be concluded that the small lymphocytes in the blood are a mixed population containing both short and long lived components.

Transfusion of Labeled Bone Marrow and Thoracic Duct Lymph.—Only those cells unidentifiable as members of a known hemopoietic series were counted in estimating the potentially effective inoculum. Cells resembling small lymphocytes made up a large portion of this group. Table V shows that relatively small numbers of labeled bone marrow cells can engender labeled macrophages in normal isologous recipients.

Of particular interest is the observation that 5% of the exudate lymphocytes in one recipient and 0.6% in another were labeled. Only a rare labeled macrophage was found in the exudates of those rats which were the recipients of labeled thoracic duct lymphocytes (Table VI). The possibility that any significant proportion of the mononuclear exudate cells could be derived from the pool of small lymphocytes having a slow turnover is opposed by the results in the recipients of small lymphocytes abundantly labeled with tritiated adenosine (Table VI).

Bone marrow appears to be a consistently good source of free macrophages (6–8). It is paradoxical that in the present experiments more lymphocytelike cells in the exudates could be derived from transfused marrow than from thoracic duct lymphocytes.

DISCUSSION

The results indicate that the majority of peritoneal macrophages which appear after i.p. glycogen injection are derived from short lived cells circulating in the blood, which are the progeny of rapidly and continuously dividing precursors present in bone marrow. These characteristics are shared by the macrophages ellicited by other inflammatory stimuli in skin and subcutaneous tissue (1, 6). It appears reasonable to assume that the peritoneal macrophages are identical with the others and that the data concerning the latter (1, 6) are applicable to the former. These include the findings that lymphocyte depletion by means of sublethal whole-body X-irradiation or prolonged drainage of thoracic duct lymph do not appreciably impair the macrophage response (6).

Since most of the peritoneal macrophages in inflammation emigrate from the blood as shown in the parabiosis experiments, the role of the milky spots of the omentum as a site of macrophage formation (9, 10) is unclear.

The relationship of exudate macrophages to the peritoneal macrophages found in normal animals has received no clarification in the present study,

although others have shown in mice (7, 8) that the "normal" peritoneal macrophages in irradiation chimeras can be derived from bone marrow.

Interesting studies on the derivation of liver macrophages in mice have recently been reported by Howard et al. (21). 11 days after the induction of graft versus host reactions with thoracic duct cells about 2% of the liver macrophages were in mitosis. Of this 2% nearly all were of donor origin. The relevance of this finding to the data presented in the present report is difficult to assess largely because of the radical differences in experimental conditions. In addition, their common property of phagocytosis need not indicate an ontogenic relationship between liver macrophages in graft against host reactions and the free macrophages in inflammatory exudates. If, for discussion's sake, it is conjectured that these are cognate cell types, there is sufficient time for an exceedingly small number of rapidly dividing precursors to generate a large number of progeny in the experiments of Howard's group. The occasional presence of macrophage precursors in thoracic duct lymph can in fact be inferred from Table VI. Neither this result nor Howard's necessitate the conclusion that macrophage formation is a common capability of thoracic duct lymphocytes.

Peritoneal washings provide a better sampling of the cells in the exudates than was possible with the glass cover slip techniques (1, 6), thus permitting the study of the so-called small lymphocytes. Although the majority of small lymphocytes in the blood and lymph are slowly produced long lived cells (16–18), the presence of at least one subpopulation of circulating lymphocytes with a fast turnover has been shown by others (22, 23). It is not known whether the fast turnover group is homogenous nor what proportion of them is capable of response to acute inflammation. The possible sources of small lymphocytes with a fast turnover include the thymus and bone marrow (17, 24, 25). Labeled macrophages could not be found in exudates either after the *in situ* labeling of the thymus in guinea pigs (26) or after the transfusion of labeled thymic cells (6) in rats. The great majority of lymphocytelike cells in bone marrow is apparently formed therein (27); their function is unknown. In the experiments being reported, infused bone marrow was shown to be a source of significant numbers of lymphocytelike exudate cells, contrasting with the largely negative yields from infusions of thoracic duct cells. The possibility of an ontogenetic association between the small lymphocytes of bone marrow and the lymphocytelike exudate cells is thus suggested by the data. In this connection others (25, 28) have attributed stem cell function to the lymphocytes formed in bone marrow but monocytogenesis has never been specifically ascribed to them. The possible role of some part of this population in the formation of blood monocytes and exudate macrophages has previously been discussed (6). This notion would appear to meet an obstacle in the clearly defined differences when the respective percentages of labeled lymphocytelike cells and macrophages in exudates are

compared at various times of harvest. In the statistical sense the populations are different; however, it can be argued that the differences may be due to the time required for one group of circulating cells to differentiate and mature. A similar argument can be invoked to explain the observed delay in emergence of newly-formed monocytes in the blood (1). Thus it may be that monocytes undergo differentiation and maturation after release from their site of production. This idea would reconcile the finding that bone marrow is the major site of monocytogenesis with the known paucity of identifiable monocytes in normal marrow.

Two other points, both elsewhere discussed (1, 6), are reinforced by the present findings. The first is that there is no apparent relationship between the large pool of recirculating small lymphocytes and mononuclear exudate cells. The second is that the term "lymphocyte" embraces cells of different origins and activities which are morphologically indistinguishable from each other.

SUMMARY

Tritiated thymidine–labeling data in individual and parabiotic rats showed that macrophages in peritoneal exudates were derived from cells in the blood which were the progeny of rapidly and continuously proliferating precursors. The characteristics of this population identify them with free macrophages studied in other sites; similarly, they can be obtained from transfused bone marrow.

Cells in the exudates which were morphologically indistinguishable from small lymphocytes were also found to have the labeling features of a rapidly proliferating population in contrast with the known kinetics of the majority of small lymphocytes in blood and thoracic duct lymph. However, experimental evidence indicated that the lymphocytelike exudate cells had emigrated from the blood and that bone marrow was a source of their precursors. These findings support the concept of the heterogeneity of lymphocytes.

The possible relationships among the mononuclear cells is discussed.

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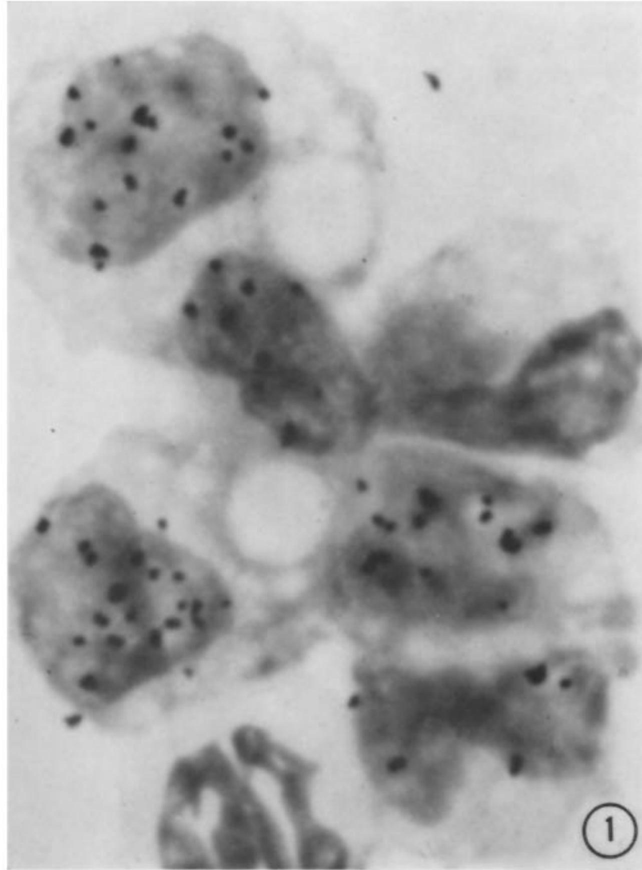
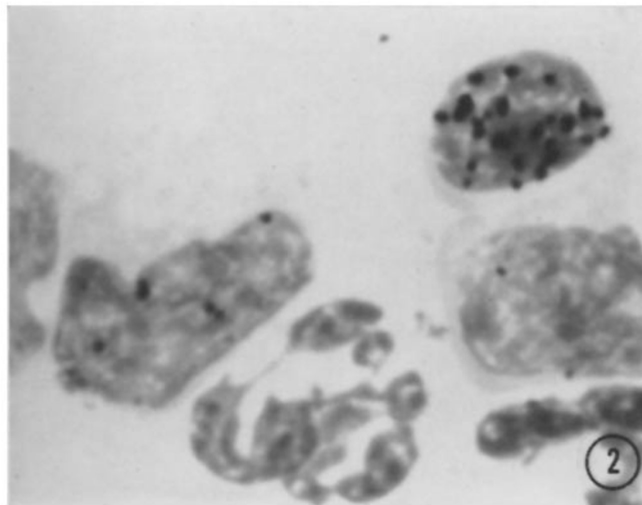
EXPLANATION OF PLATE 19

Labeled mononuclear cells in peritoneal exudates of 24 hr duration. A single injection of $TdR-H^3$ was given intravenously 48 hr before harvest.

FIG. 1. Labeled macrophages. May-Gruenwald Giemsa, × 3000.

FIG. 2. A heavily labeled "small lymphocyte" is seen to have typical dense, clumped nuclear chromatin and scant cytoplasm. May-Gruenwald Giemsa, × 3000.





(Volkman: Mononuclear cells in peritoneal exudates)