CHARACTERIZATION OF NONLYMPHOID CELLS DERIVED FROM RAT PERIPHERAL LYMPH

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Mammalian peripheral lymph contains cells that are distinct from lymphocytes and were considered to be macrophages because of their irregular surface morphology (1). Recent studies in the rabbit and pig (2, 3) suggest that many of these cells are not typical macrophages, being nonphagocytic and showing different nonspecific esterase (NSE)[1] activity. In dermal lymph they may be related to Langerhan's cells. Migration of these cells from tissues to lymph nodes suggests that they may act as accessory cells in antigen transport and presentation (4). Analysis of this possibility has been hampered by the need to study these cells in large animals, where inbred strains are not available.

Following mesenteric lymphadenectomy in rats, cells with irregular surface morphology appear in thoracic duct lymph (G. Mayrhofer, unpublished observations). We describe this model and show that nonlymphoid cells (NLC) derived from peripheral lymph can be collected for several days. We characterize freshly collected NLC in terms of morphology, cytochemistry, density, surface phenotype, and endocytosis and examine their local and distant origins and kinetic properties.

A few NLC resemble mononuclear phagocytes. The majority differ in many ways from macrophages but are themselves heterogeneous. The differences may be maturational or reflect the presence of distinct lineages.

Materials and Methods

Animals. Hooded (PVG/c), Agouti (DA), albino (AO), PVG/1a, and PVG-RT1^u strain rats and the F1 hybrids, (PVG/c × DA) and (PVG/c × PVG-RT1^u), were bred under specific

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[1] *Abbreviations used in this paper:* Ag, antigen; ATPase, adenosine triphosphatase; C'R, complement receptor; DAB, Dulbecco's A + B salt solution; E, sheep erythrocytes; EA, antibody-coated sheep erythrocytes; EIgM, IgM antibody-coated sheep erythrocytes; EIgMC, IgM antibody- and complement-coated sheep erythrocytes; EM buffer, 0.1 M sodium cacodylate containing 1% sucrose and 2 mM calcium chloride at pH 7.4; FcR, receptor for the Fc portion of immunoglobulin; FCS, fetal calf serum; [^{3}H]TdR, tritiated thymidine; IgMαE, IgM fraction of rat anti-sheep erythrocyte serum; MEMS, Eagle's minimal essential medium + 10% FCS; MFR, lectinlike receptor with specificity for mannose and fucose residues; MLR, mixed leukocyte reaction; NCS, newborn-calf serum; NLC, nonlymphoid cell; NSE, nonspecific esterase; PBS, phosphate-buffered saline; RPC, resident peritoneal cells; SEM, scanning electron microscopy; sIg, membrane immunoglobulin; TDL, thoracic duct leukocytes from normal donors; TEM, transmission electron microscopy; X.TDL, thoracic duct leukocytes from mesenteric lymphadenectomized donors; $^{\gamma}$X.TDL, thoracic duct leukocytes from mesenteric lymphadenectomized donors that were sublethally irradiated immediately before cannulation.

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pathogen-free conditions in the MRC Cellular Immunology Unit, Oxford. Outbred and DBA/2 strain mice were bred in this laboratory.

Animal Procedures

MESENTERIC LYMPHADENECTOMY. 5-wk old rats were injected with 0.1 cm^3-Synkavit 10 (Roche Products Ltd., Welwyn Garden City, England). 7 d later all caecal, mesenteric, portal, and pancreatic lymph nodes (5) were removed by blunt dissection. Animals were allowed to recover for at least 6 wk before use.

THORACIC DUCT CANNULATION. Thoracic duct cannulation was performed as described by Ford (6).

CANNULATION OF PERIPHERAL LYMPHATICS. Lacteal lymph samples were collected and quantitated as described by Steer (7) and stained for NSE.

ANIMAL IRRADIATION. Animal irradiation was performed at 1.67×10^{-2} $Gy \cdot s^{-1}$ using a ^{137}Cs source of γ rays (Gamma cell 40, Atomic Energy Commission of Canada Ltd.). When NLC from irradiated donors were compared with other cells the donors of these cells were similarly irradiated.

BONE MARROW CHIMERAS. Lymphadenectomized PVG/c rats were irradiated with 10 Gy and given 6×10^7 viable (PVG/c × DA)F1 bone marrow cells intravenously. By 21 wk their blood leukocyte counts were normal. Chimerism was analyzed by examining autoradiographically the binding of the DA strain-specific monoclonal antibodies R2/15S and R3/13 (8) to their X.TDL. X.TDL from (PVG/c × DA) F1 and PVG/c rats served as controls.

ALVEOLAR CELLS. Chloral hydrate-anesthetized rats were killed by exsanguination. The trachea was cannulated, the thorax opened, and the lungs lavaged three times with 0.9% wt/vol sodium chloride solution. The washes were pooled to yield $\sim 3 \times 10^6$ alveolar cells per rat.

RESIDENT PERITONEAL CELLS (RPC). RPC were harvested in 15 cm^3 of ice-cold sterile phosphate-buffered saline (PBS) containing 20 IU heparin per cm^3. Yields were $\sim 10^7$ cells per rat.

TRITIATED THYMIDINE ([^{3}H]TDR) LABELING IN VIVO. Animals were injected intravenously with [^{3}H]TdR (1 μCi/gm body weight, TRK-61, Radiochemical Centre, Amersham, England). Samples were either left unstained or stained for NSE and coated with 0.5% gelatin to avoid negative chemography. They were then coated with Ilford K5 emulsion and exposed for 2–4 wk.

Handling of Cell Suspensions. Handling of cell suspensions and washing was generally in PBS + 2% NCS or PBS + 0.1% bovine serum albumin maintained at 4°C. 10 mM azide was added to prevent capping of surface antigens.

Erythrocytes were lysed by 10 min of treatment at room temperature with 0.14 M ammonium chloride in 0.17 M Tris-HCl, pH 7.2 (NH_4Cl-Tris).

Cells were cultured in Eagle's minimal essential medium plus 10% fetal calf serum (MEMS) or RPMI 1640 plus 10% heat-inactivated rat serum at 37°C in 5% CO_2 in air.

When different cell populations were compared they were subjected to identical treatments.

Quantitation of Cells. Cell counts were made using a Coulter Counter model Fn (Coulter Electronics Ltd., Harpenden, UK). Differential counts were made on cytocentrifuge preparations stained with Giemsa or for NSE and also on live cells using Nomarski interference or phase optics.

[^{3}H]TdR Labeling In Vitro. Washed X.TDL were incubated for 1 h at 37°C in MEMS plus 1 $\mu Ci \cdot cm^{-3}$ [^{3}H]TdR. After three washes, autoradiographs were made and developed after 2–4 wk.

Electron Microscopy. Washed X.TDL were fixed for 0.5 h at 4°C in 2.5% glutaraldehyde (Agar Aids, Stansted, England) in 0.1 M sodium cacodylate containing 1% sucrose and 2 mM calcium chloride at pH 7.4 (EM buffer). To preserve surface morphology, lymph sometimes was dripped directly into gently stirred fixative maintained at 30–37°C in a water bath.

Cells were washed twice with EM buffer and postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.4 for 50 min. For transmission microscopy (TEM), cells were dehydrated and embedded. Thin sections were stained with lead citrate (9) and examined in a JEOL 100 XC electron microscope.

For scanning electron microscopy (SEM) fixed cells were either allowed to adhere to poly-L-lysine coated glass strips (10) or deposited onto Millipore (Millipore Corp., Bedford, MA)

filters (3). Washed cells were sometimes allowed to sediment directly onto glass slips. After 1–2 h incubation at 37°C in MEMS in 5% CO_2, nonadherent cells were removed and the adherent cells fixed in glutaraldehyde and processed.

After critical-point drying and sputter-coating with gold (Polaron Ltd., Watford, UK) samples were examined in the JEOL 100 XC with ASID attachment.

Density Gradients. Linear and step gradients of Percoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden) in divalent cation-deficient Hank's solution were spun (600 *g*, 30 min) and fractionated at 4°C. The linearity of gradients was assessed by measuring the refractive indices of fractions collected. Density and refractive index were linearly related (results not shown).

The total number of cells per gradient was less than that at which streaming might occur.

Fractions were also examined by SEM or light microscopy.

Phagocytosis

OPSONIZED ERYTHROCYTES (EA). Sheep erythrocytes (E) were incubated for 0.5 h with subagglutinating dilutions of rat anti-E antibody, washed three times in PBS and added at a concentration of 2.0% to X.TDL in suspension, or after adherence to glass coverslips. Samples were incubated for 0.5–2 h at 37°C. Noningested E were sometimes lysed using NH_4Cl-Tris or removed from adherent preparations using distilled water. Samples were then stained for NSE.

LATEX. X.TDL was cultured for 0.5–1 h with various concentrations of washed 0.81 μm particles (Difco, E. Molesey, Surrey, UK). FCS was added to a concentration of 10% and incubation continued for 0.5 h. The cells were washed and prepared for light or electron microscopy.

Pinocytosis. Cells were incubated at 4°C or 37°C for 45 min with 1 $mg \cdot cm^{-3}$ ferritin (Calbiochem, CP Laboratories, Bishop's Stortford, Hertfordshire, UK) washed and incubated at the same temperature for 5 min. This did not affect the ability of cells to exclude trypan blue. Samples were then prepared for TEM.

Fc Receptor (FcR) Assays

IN SUSPENSION. The method was based on that of Parish and Hayward (11).

PANNING. Panning was a modification of the method of Wysocki and Sato (12). Bacterial grade petri dishes (Sterilin Ltd., Teddington, UK) coated with ovalbumin or ovalbumin antiovalbumin complexes were washed three times with PBS. Subsequent procedures were performed at 4°C. Cells were added to test and control plates and allowed to settle for 2 h. Nonadherent cells were gently washed off, counted, and cytocentrifuged. The plates and cytocentrifuge preparations were NSE stained.

Rat anti-E IgM (IgMαE). Rat serum prepared 5 d after intravenous injection of 10^8 washed E was fractionated on a Sephacryl S-300 column. E were opsonized with antibody and complement (EIgMC) or antibody alone (EIgM) by incubation with serum fractions and then with or without fresh DBA/2 mouse serum (C'5-deficient). A fraction causing EIgMC rosette formation on adherent thioglycollate-elicited mouse peritoneal cells at a dilution of 1:320, but no EIgM rosetting at a dilution of 1:2, was designated IgMαE and used in the complement receptor assay.

Complement Receptor (C'R) Assay. EIgMC, EIgM, or E prepared as above, were mixed with leukocytes, resuspended in ≃20 μl of medium and centrifuged, and incubated for 0.5 h. Rosettes were then scored.

Mannose/Fucose Receptor (MFR) Assay. Binding assays using ^{125}I-mannose bovine serum albumin were performed in the presence and absence of yeast mannan, a competitive inhibitor (13).

Autoradiography. Cells were labeled at 4°C under saturating conditions with antibody (see text), washed twice, and incubated with ^{125}I-labeled (14) antibody specific for the first antibody. After three washes cytocentrifuge preparations were made (Shandon Southern Instruments, London), stained for NSE and methanol-fixed. Slides were subbed, dried, dipped (K2 emulsion; Ilford Ltd., Liverpool, UK), and then developed after 2–10 d.

Removal and Resynthesis of Surface Immunoglobulin (sIg). X.TDL were incubated in 0.1% pronase (Calbiochem) in Dulbecco's salt solution (DAB) for 0.5 h at 37°C and washed twice in DAB plus 10% FCS. Treated and control cells were cultured overnight, harvested, and examined for sIg (see above).

Cytochemistry

NONSPECIFIC ESTERASE. Dried preparations were fixed for 60 s in formalin vapor. NSE was shown using α-naphthyl butyrate and hexazotized pararosanaline in cacodylate buffer, pH 6.0 (15). Cells were stained at room temperature for 1, 5, or 30 min.

Controls omitted substrate or coupling agent. Preincubation of samples for 45 min with 0.01 or 0.04 M sodium fluoride and incubation in reaction mixture containing fluoride at the same molarity was also performed.

ACID PHOSPHATASE. The technique of Lewis and Knight (16) was used. Controls included incubation with reaction mixture lacking substrate.

DNA. Samples fixed in 10% buffered formalin or in methanol for 10 min were stained by the Feulgen technique (17). Hydrolysis was omitted in controls.

ADENOSINE TRIPHOSPHATASE (ATPASE). The method of Robins and Brandon (18) was used. Controls consisted of incubation in reaction mixture lacking ATP or divalent cations. In the latter case substrate was made up without adding magnesium sulphate and 0.1 M EDTA was added to chelate remaining divalent cations.

PEROXIDASE. Light microscope samples were fixed for 60 s in formalin vapor and those for TEM were glutaraldehyde-fixed and washed. Samples were incubated for 10–30 min in 1 mg·cm^{-3} diaminobenzidine (Polysciences, Inc., Warrington, PA) in 0.05 M Tris-HCl with 0.005% hydrogen peroxide (19) and counter stained or processed for TEM. Controls omitted hydrogen peroxide from the reaction mixture.

Results

Mesenteric Lymphadenectomy. To check the lymphadenectomy, six rats were injected postoperatively with pontamine sky blue and examined 7 d later. In only one animal was one mesenteric node seen. No regeneration of nodes was seen up to 2 yr after surgery.

Apart from slightly raised nucleated blood cell counts and minor intraperitoneal adhesions, the only obvious complication was that 5/285 animals developed intestinal distension, possibly due to adhesions or damaged autonomic nerves.

Morphology of NLC. Results of light and electron microscopy were essentially similar and are integrated in the following description of NLC. Their diameter was 10–16 μm.

PLASMA MEMBRANE. Plasma membrane was always extremely irregular and thus NLC were easily distinguished from lymphoid cells. ~20% of NLC possessed smooth, thin (0.2 μm) surface folds or veils (Fig. 1, top), extending up to 10 μm from the cell body and being largely free of organelles. 50% of NLC possessed many blunt pseudopodia of varying lengths (Fig. 1, bottom). Many were short (1–3 μm), knoblike projections densely packed on the cell surface. Many cells had long irregular pseudopodia extending 10–12 μm from the cell body (Fig. 2, top left). Occasional cells clearly possessed both blunt pseudopodia and extensive veils (Fig. 2, top right).

Live NLC allowed to settle on surfaces exhibited fine pseudopodia, which were apparently adherent (Fig. 2, bottom left). In contrast some cells spread rapidly (Fig. 2, bottom right). The frequency in X.TDL of such cells increased greatly when the rats were conventionally housed.

CYTOPLASM. NLC possessed much electron-lucent cytoplasm. Mitochondria were present in moderate numbers, 85% being small, with round or oval profiles. The Golgi apparatus was conspicuous with multiple areas often present in one section of a cell. Many vesicles were seen, some near the Golgi apparatus, but others close to the cell surface. These may be endocytic or secretory. Rough endoplasmic reticulum was always present, often as parallel arrays. Rough and smooth endoplasmic reticulum

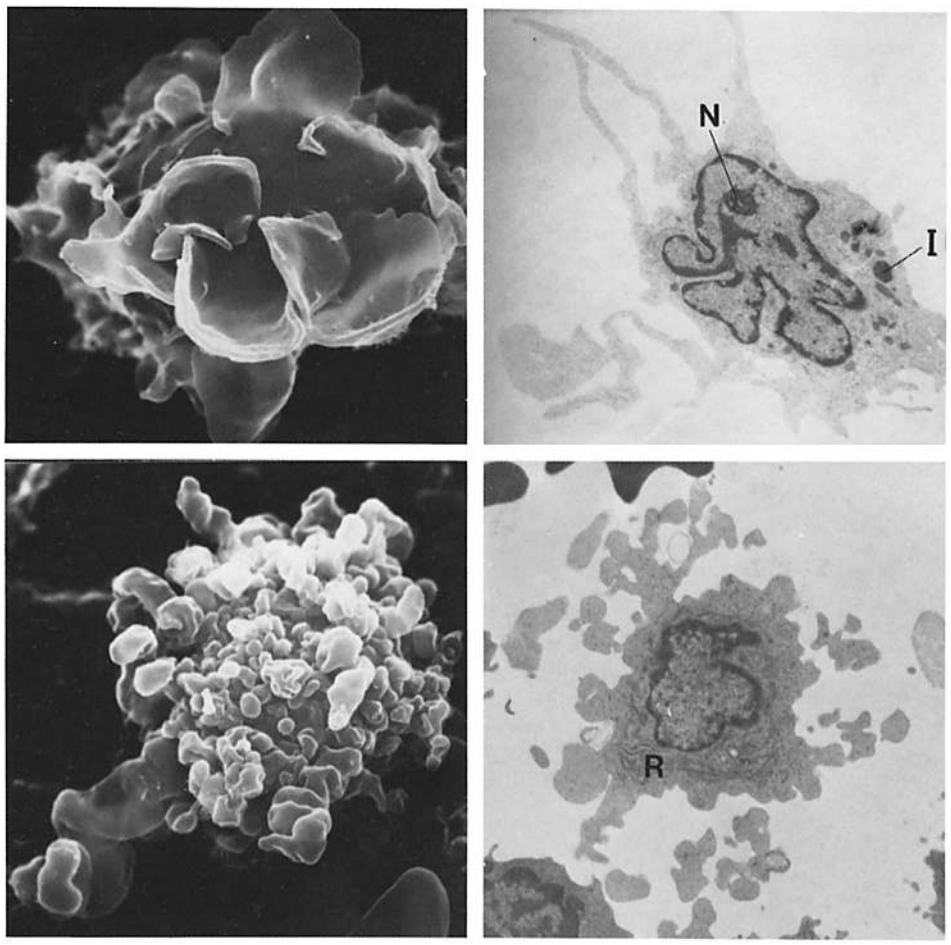

FIG. 1. NLC viewed by SEM and TEM. (Top left) Small NLC showing thin veils. SEM magnification × 8,600. (Top right) NLC with several veils cut transversely. The nucleus is folded and contains a nucleolus (N). The cytoplasm contains a large inclusion (I). TEM magnification × 3,300. (Bottom left) NLC displaying numerous blunt pseudopodia of different lengths. SEM magnification × 6,500. (Bottom right) NLC with blunt pseudopodia and conspicuous rough endoplasmic reticulum (R). TEM magnification × 5,400.

sometimes extended into the pseudopodia. A few polyribosomes were present. Bundles of microfilaments were frequently observed parallel to the nuclear membrane.

20–39% of NLC contained conspicuous 0.1–6 μm diameter cytoplasmic inclusions. Some were homogeneous but others contained recognizable cellular debris, including mitochondria. A proportion of inclusions contained acid phosphatase, nonspecific esterase, peroxidase, and DNA.

Birbeck (Langerhan's cell) granules (20) were not seen in NLC.

NUCLEUS. The nucleus was usually irregular in outline and was frequently situated eccentrically. The amount of heterochromatin was variable. A nucleolus was present in many NLC (Fig. 1, top right).

Cytochemistry of NLC

NONSPECIFIC ESTERASE. >90% of cells identified as NLC by their morphology gave positive reactions. Three categories were classified: (*a*) 3+ cells (≃40% of NLC) stained

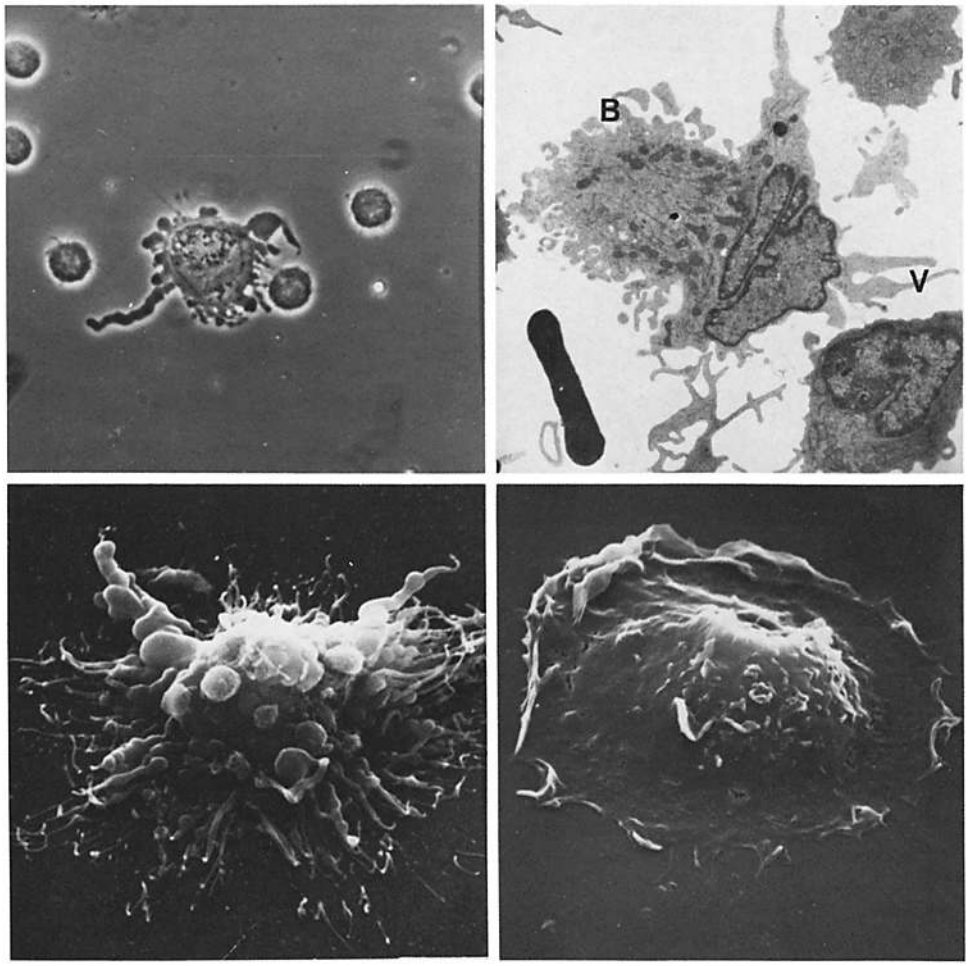

FIG. 2. (Top left) Living NLC possessing both blunt and fine pseudopodia. Phase contrast. × 1,040. (Top right) NLC possessing both veils (V) and blunt pseudopodia (B). The nucleus is indented. The cytoplasm is relatively abundant. TEM magnification × 4,000. (Bottom left) Adherent NLC possessing blunt pseudopodia extending from its upper surface and fine pseudopodia in close contact with the substrate. SEM magnification × 4,300. (Bottom right) Adherent NLC displaying spreading. SEM magnification × 6,480.

so intensely after 30 min of incubation that the reaction product totally obscured their morphology (Fig. 3, top left). Moderate reactivity was seen after 1 min. The reaction product was clearly located in the cytoplasm as discrete circular deposits (Fig. 3, top right). This distribution was confirmed on 1 μm sections of X.TDL. (*b*) 2+ cells (≃30% of NLC) stained strongly for NSE in the perinuclear area (Fig. 3, bottom left). (*c*) 1+ cells (≃30% of NLC) showed similarly distributed, but much weaker reactivity. Controls stained in the presence of sodium fluoride showed no specific reactivity.

ENDOGENOUS PEROXIDASE. At all times after cannulation 20–30% of NLC stained for peroxidase. 70% of positive cells showed fine granular reactivity similar to that of blood monocytes. These NLC were generally small. 30% of positive NLC contained

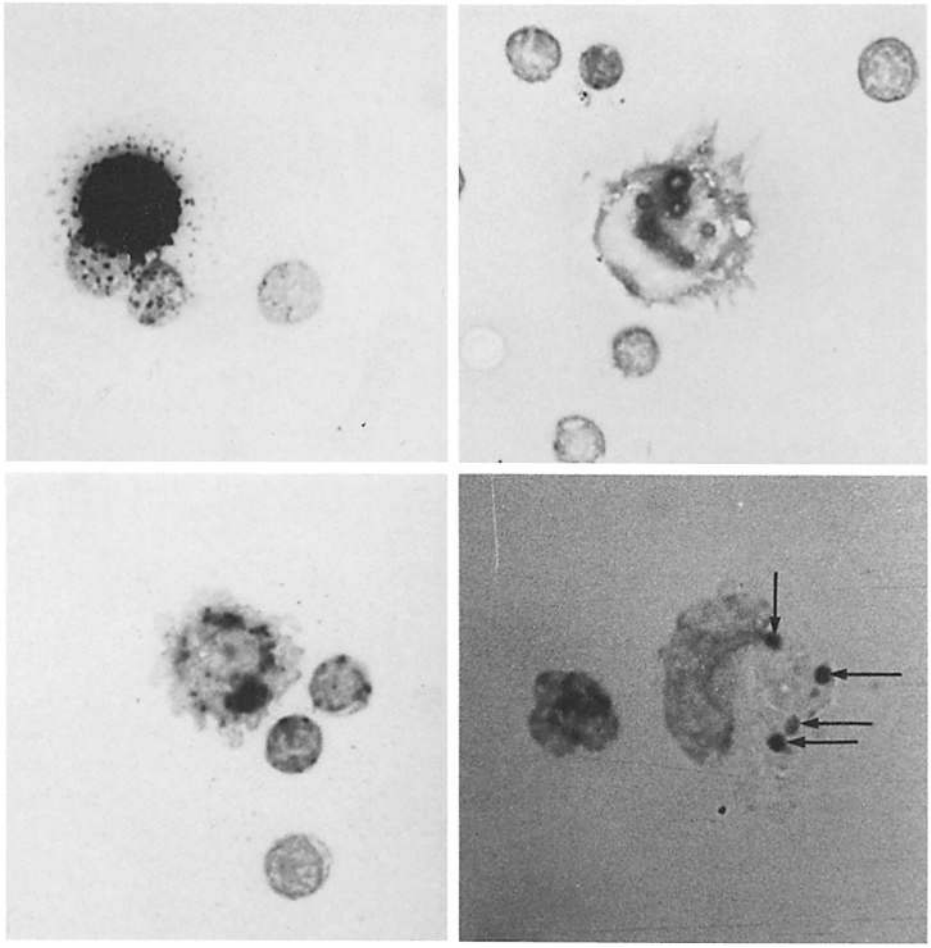

FIG. 3. (Top left) Cytocentrifuge preparation of X.TDL stained for NSE for 30 min. An NLC (3+) is obscured by reaction product which is also affecting two adjacent lymphocytes. × 1,500. (Top right) Similar preparation stained for 1 min. A large NLC shows reaction product in discrete locations within the cytoplasm. × 1,500. (Bottom left) Similar preparation stained for 30 min. An NLC (2+) contains discrete but intense areas of cytoplasmic reaction product. × 1,500. (Bottom right) A Feulgen-stained NLC showing positive cytoplasmic inclusions (→). × 2,000.

one or more large dense inclusions. These cells were large. No reactivity was observed in the absence of hydrogen peroxide.

ACID PHOSPHATASE. Acid phosphatase was seen in some inclusions in NLC, indicating their lysosomal nature. No label was deposited on samples incubated in reaction mixture lacking substrate.

FEULGEN REACTIVITY. 8–18% of NLC contained Feulgen-positive cytoplasmic inclusions up to several micrometers in diameter (Fig. 3, bottom right). Controls omitting hydrolysis were negative.

ATPASE. Prominent ATPase was present near the surface of all NLC. This labeling did not occur in the absence of ATP or divalent cations.

Density of NLC. X.TDL were fractionated on linear Percoll gradients (Fig. 4) with overall recoveries of 80–100%. NLC recovery was 60–100%, showing that a slight

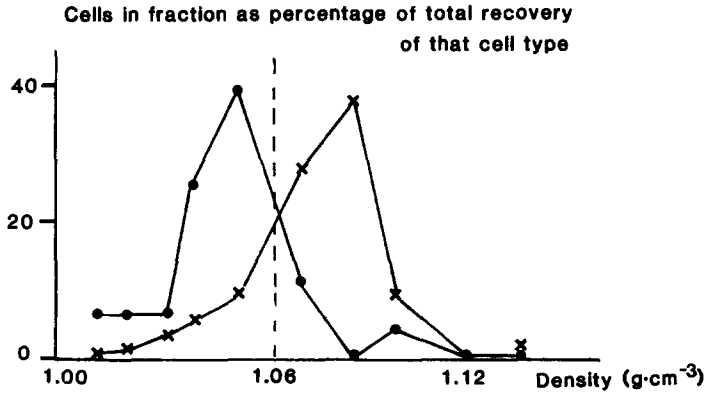

FIG. 4. X.TDL was separated on a linear Percoll gradient spun to equilibrium. Profiles of NSE positive cell recovery (●) and total cell recovery (×) at different densities are shown.

selective loss often occurred.

Step gradients of a layer of 45% Percoll below a layer of 9% Percoll allowed 6–17-fold enrichment of NLC from X.TDL. Using X.TDL from sublethally irradiated rats (ϟX.TDL), such gradients allowed the preparation of suspensions containing ≃80% NLC. NLC prepared by this technique lost their normal surface morphology. However, a full range of morphologies reappeared if cells were cultured briefly after separation.

Adherence. After adherence the majority of NLC were removed by vigorous washing; those remaining were small and stained weakly for NSE (± − 1+). With gentle washing some larger NSE > 1+ NLC remained but the number of lymphocytes remaining increased greatly. In contrast to their poor adherence to glass or plastic, NLC often formed spontaneous rosettes with lymphocytes.

Phagocytosis

EA IN SUSPENSION. Incubation of X.TDL with EA in suspension showed that large NSE 1+ − 3+ NLC did not phagocytose (<1%), although occasional small monocytoid NLC internalized EA. These cells were either weakly (±) positive or negative for NSE and were repeatedly seen in close contact with NSE 2+ − 3+ NLC.

EA ON ADHERENT CELLS. Only small numbers of NLC adhered firmly to glass (see preceding section). 60–70% of such cells internalized EA.

LATEX. After incubation of X.TDL in suspension with latex beads, NSE negative or 1+ small NLC were associated with large numbers (>30), whereas larger NSE 2+ − 3+ NLC were associated with small numbers (2–10). TEM was used to clarify whether these particles were internalized. In monocytoid NLC many latex particles were in membrane-bounded vesicles, some of which contained peroxidase. Most of the larger NLC did not include latex particles. Occasionally one or two internalized particles were seen.

Pinocytosis by NLC. X.TDL and RPC were incubated with ferritin. By TEM no ferritin was seen associated with either cell type maintained at 4°C indicating that there was no passive adsorption. At 37°C no NLC took up ferritin although a few granulocytes present in this sample were labeled. In contrast, 66% of the RPC internalized ferritin, the negative cells being lymphocytes.

Expression of FcR. The poor adherence of NLC precluded FcR assays involving

adherent monolayers. Rosetting using EA in suspension indicated that a few NLC possess weak FcR activity (Table I). The adherence of cells to plates coated with immune complexes bearing exposed Fc regions, and to plates coated with antigen was

TABLE I
FcR in Suspension

Cell source	Treatment	Number of NSE positive cells with:			Number of SRBC per 100 NSE positive cells
	SRBC:	0	1–5	>5	
Alveolar macrophage	EA	55	33	27	320
	E	83	17	0	21
RPC	EA	25	70	22	336
	E	81	18	0	40
X.TDL	EA	49	50	1	112
	E	75	24	1	49

Cells were incubated in suspension for 1 h at 37°C with erythrocytes (E) or antibody-coated erythrocytes (EA). The number of erythrocytes associated with each of 100 NSE-positive leukocytes was determined in each sample. No distinction was made between erythrocytes bound and internalized.

TABLE II
Panning for Cells with FcR

Cell source	Plate coated with (+) or without (−) antibody	Percent of cells added that adhered to plate	Percent composition of nonadherent cells (samples of 500)		Composition of adherent cells (counts per unit area of plate)	
			NSE^+	NSE^-	NSE^+	NSE^-
Alveolar macrophage	+	95.5	85	15	738	22*
	−	2.2	79	21	8	2
RPC	+	30	11	89	330	67*
	−	0	45	55	5	3
ϟX.TDL	+	10	24	76	3	59*
	−	6.7	19	81	2	29*

* Many of these cells were granulocytes or appeared dead.
Results obtained when a modification of the "panning" technique described by Wysocki and Sato (12) was used to compare the ability of cells to adhere at 4°C to plates coated with ovalbumin antiovalbumin immune complexes (+) bearing exposed Fc regions, and to plates coated with ovalbumin alone (−). 4.5 × 10^5 alveolar cells, 10^6 RPC, and 3 × 10^6 ϟX.TDL were added per plate.

TABLE III
Rosetting Assay for C'R

Ligand	Cell type	Number of leukocytes with:	
		<3 SRBC	>3 SRBC
EIgMC	NLC	45	5
	lymphocytes	331	169
EIgM	NLC	50	0
	lymphocytes	498	2
E	NLC	50	0
	lymphocytes	ND	ND

Cells and ligands were incubated together for 0.5 h and binding was assessed.
The lymphocytes present in suspension with the NLC were used as an internal positive control. ND, not done.

TABLE IV

Binding Assay to Study Expression of Mannose R

Cell source	Dose of ^{125}I-mannose BSA $\mu g \cdot cm^{-3}$	cpm bound (mean of triplicates)		Difference (specific binding)
		ligand alone	ligand + 5 $mg \cdot cm^{-3}$ mannan	
ϕX.TDL	4	10,571	10,614	—
	1	2,736	2,522	214
Alveolar macrophages	4	28,418	5,190	23,228
	1	12,056	2,246	9,810

Binding assays were performed using ^{125}I-mannose BSA, in the presence and absence of the competitive inhibitor, yeast mannan, to examine leukocytes for MFR. Binding of the iodinated ligand to populations of alveolar cells and of ϕX.TDL containing similar numbers of macrophages and NLC was compared.

TABLE V

Analysis of Surface Markers by Autoradiography

First antibody	Specificity	Grains per 100 NSE positive cells	P_s	Percent of positive lymphocytes
MRC OX1 (21)	Rat leukocyte common antigen	>600	1	99
MRC Ox6 (22)	Rat equivalent of mouse I-A subregion product	>600	1	46
MRC Ox8 (23)	Rat Tc/s and thymocyte subset	147	0	12
MRC Ox12 (24)	Rat kappa chain	251	0.26	48
MRC Ox19 (25)	All rat T cells and thymocytes	151	0	50
W3/13 (26)	All rat T cells and thymocytes plus granulocytes and plasma cells	305	0.34	49
W3/25 (27, 28)	Rat T_H and thymocyte subset, rat macrophages	191	0	38
W6/32 (29)	Common HLA determinant	173	—	0

Cells were labeled in suspension indirectly with the mouse monoclonal antibody specified followed by ^{125}I-labeled rabbit anti-mouse Ig under saturating conditions. Autoradiographs were prepared and grain counts were performed. The proportion of NLC specifically labeled (P_s) was determined using the method of England, Rogers, and Miller (30). Labeling by the monoclonal antibodies was controlled by checking the percentage of labeled lymphocytes (counts of 500 cells). Binding to rare granulocytes in X.TDL was also as expected.

compared (Table II). NLC lack levels of FcR comparable to those on macrophages and granulocytes used as positive controls. Working at 4°C successfully prevented nonspecific adherence of cells to the plates.

Expression of C'R. A rosetting assay (Table III) showed that NLC lack C'R, whereas they are clearly present on some lymphocytes.

Expression of MFR. Comparison was made between the binding of ^{125}I-mannose BSA, with or without a competitive inhibitor, to populations of alveolar cells and of ϕX.TDL in which the numbers of macrophages and NLC present were comparable (Table IV). Ligand bound specifically to alveolar cells but not to ϕX.TDL. Therefore no single-cell analysis was performed.

Surface Markers of NLC Defined by Monoclonal Antibodies. In a typical experiment (Table V) NLC express the leukocyte common antigen and Ia antigens but not W3/25. NLC are bound by MRC Ox 3, 4, and 6, which recognize the rat equivalent of mouse I-A subregion antigens (22, 31). Binding is abolished by preincubating the

antibodies with pure antigen. NLC also label with MRC Ox 17 antibody, directed against I-E subregion antigens (31) (not shown). W3/13 and MRC Ox12 (anti-kappa chain) bind slightly to NLC. NLC do not express the other lymphocyte antigens tested.

Quantitative Estimation of the Expression of Ia Antigens by NLC. The amount of Ia antigen per NLC was estimated by comparing autoradiographically binding of MRC Ox 6 under saturating conditions to NLC and B cells. In two experiments the ratios of specific grains per NLC to specific grains per positive lymphocyte were found to be 10.3 and 10.1. Therefore NLC bear $\sim 1.5 \times 10^6$ Ia antigens per cell. Similar results were obtained using MRC Ox4. The response of the emulsion to increasing amounts of radioactivity was linear over the range used.

Origin and Nature of sIg on NLC. Some NLC bear low levels of sIg. The proportion of such NLC varied. Autoradiography using rabbit anti-rat Ig class-specific reagents (32) (from Dr. A. F. Williams) indicated that the sIg on NLC was of more than one class (results not shown). It was not established whether an individual NLC carried sIg of more than one class.

Pronase treatment of X.TDL in which 56.2% of NLC possessed sIg reduced the percentage of labeled cells to 3.5%. Of NLC recovered after overnight culture only 6.0% were labeled, indicating that NLC cannot synthesize Ig and that the sIg is cytophilic.

Kinetic Properties of NLC

OUTPUT (FIG. 5*a*). NLC comprised 0.5–4% of X.TDL. NLC output remained relatively constant for 5–6 d at $1.5–2.5 \times 10^5$ cells/h. As lymphocyte output fell the percentage of NLC increased.

EFFECTS OF IRRADIATION (FIG. 5*a*). After 5.0 Gy irradiation lymphocyte output fell very rapidly, but NLC output was not significantly affected for 3 d. Thus, the proportion of NLC was $\simeq$10% on day 1, 20–30% on day 2 and 20–50% on day 3.

PRESENCE OF NLC IN NORMAL TDL. TDL from normal rats with and without 5.0 Gy irradiation were NSE stained. NLC were always seen in TDL from the unirradiated rats at 0.017–0.1% (i.e., $0.5–3 \times 10^4$ NLC/h). 2 d after irradiation their frequency peaked at $\simeq$10%. Some NLC contained inclusions, sometimes recognizable as cells.

DNA SYNTHESIS BY NLC IN VITRO. Large lymphocytes, but not NLC were labeled with [^{3}H]TdR.

OUTPUT OF LABELED NLC AFTER INTRAVENOUS [^{3}H]TDR. Rats were injected with [^{3}H]TdR immediately after cannulation. Incorporation by X.TDL was assessed by autoradiography (Fig. 5*b* and *c*). The proportion of labeled NLC was 1.25% after 24 h, peaked at 24% on day 4 and was 16% by day 6.

At 24 h the only labeled NLC are NSE 1+ (Fig. 5*c*). The labeling index of NSE 1+ NLC rose to a maximum of 40% at 72 h and fell to 20% by day 5. NSE 2+ and 3+ NLC had similar labeling kinetics, the first labeled cells appearing at 48 h and peak labeling (15–20%) occurring on days 3–5. These labeling patterns were distinct from those of lymphocytes.

Local Origin of NLC

PERITONEAL CELLS. To determine whether peritoneal cells can enter X.TDL, syngeneic RPC were labeled in vivo and transferred orthotopically into cannulated lymphadenectomized rats.

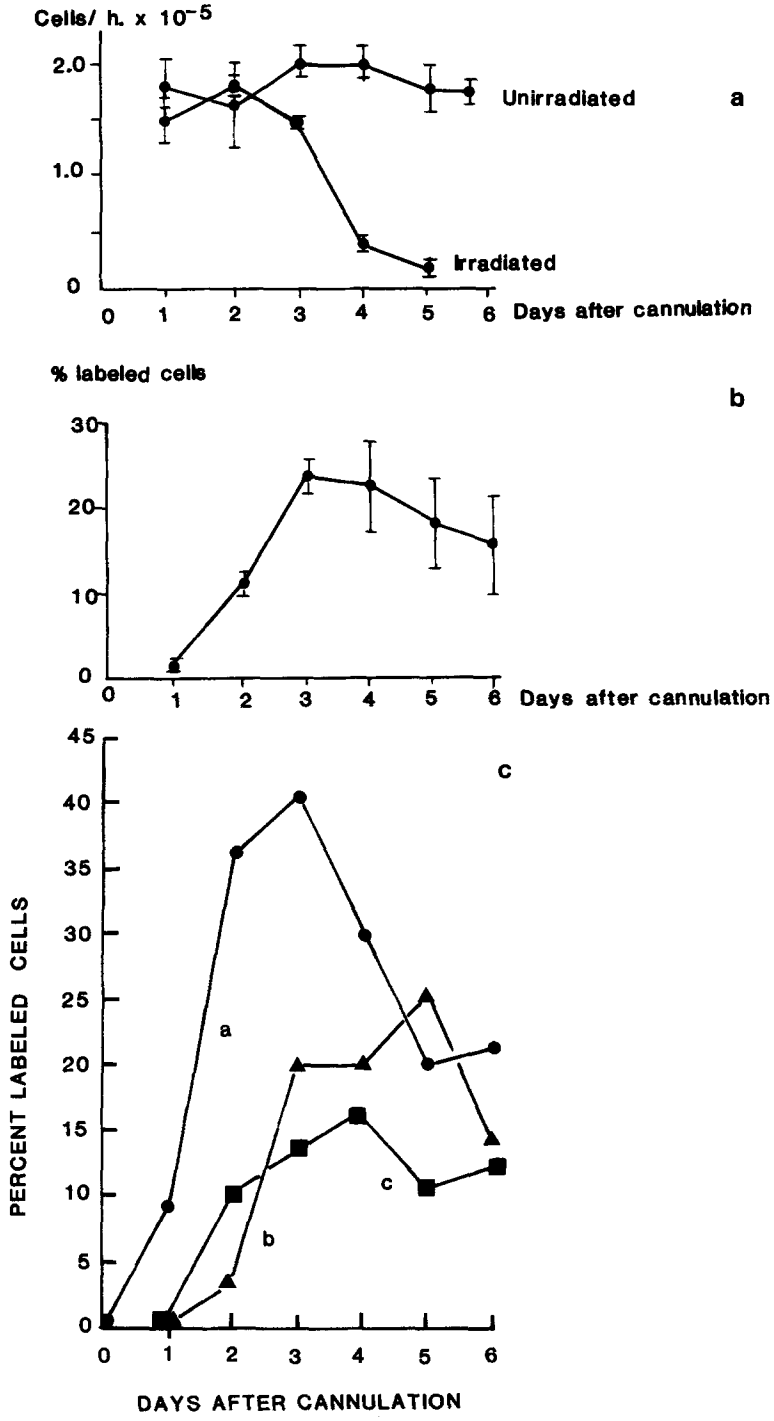

FIG. 5. (*a*) Output of NLC into X.TDL with and without irradiation. Points are means from six animals ± 1 SEM. (*b*) Labeling index of NLC in X.TDL at intervals after giving [^{3}H]TdR intravenously. Points are means from four animals ± 1 SEM. (*c*) Labeling index of NLC in X.TDL classified by NSE staining at intervals after giving [^{3}H]TdR intravenously. Points are means from four rats. *a*, NSE 1 + NLC; *b*, NSE 2 + NLC; *c*, NSE 3 + NLC.

TABLE VI

Relationship between NLC in X.TDL and RPC

Overnight collection	Proportion of NLC bearing carbon	
	Rat A	Rat B
1	4/153 (2.6)	6/212 (2.8)
2	3/230 (1.3)	ND
3	13/230 (5.6)	2/40 (5)
4	4/200 (2.0)	4/200 (2.0)
5	1/149 (0.7)	ND

Isogeneic carbon labeled RPC were adoptively transferred into the peritoneal cavities of each of two recently cannulated mesenteric lymphadenectomized rats. Each rat received a total of 1.7×10^7 RPC of which 56% were carbon labeled and 96% excluded trypan blue. The output of X.TDL from these rats was monitored for the presence of carbon-labeled NLC. Figures are the number of carbon-labeled NLC observed divided by the total number of NLC examined. The figures in brackets are the same data expressed as a percentage. ND, not done.

TABLE VII

Bone Marrow Origin of NLC by Autoradiography

Rat cells screened as:	(PVG/c × DA)F1 → ↯PVG/c		(PVG/c × DA)F1		PVG/c	
	+	−	+	−	+	−
NLC	16	0	16	0	0	10
Lymphocyte	943	16	962	9	1	985
Granulocyte	25	0	13	0	0	4
Total	984	16	991	9	1	999

Samples from the sources indicated were assessed for chimerism at the single cell level by autoradiography of NSE-stained cytocentrifuge preparations using an indirect labeling regime consisting of incubation with the DA strain-specific monoclonal antibodies R2/15S and R3/13 (8) followed by ^{125}I-labeled subclass-specific second antibody. A clear distinction could be made between positive cells (8 or more grains) and negative cells (2 or fewer grains). 1,000 cells from each of the specified sources were scored as positive or negative for the binding of DA strain-specific monoclonal antibodies and classified as one of three cell types on the basis of their morphology.

Table VI shows that a few phagocytic RPC can enter X.TDL. Most labeled cells were monocytoid and only a very few typical NLC were labeled. The total output of NLC from recipient rats was similar to that in uninjected controls (data not shown).

NLC IN INTESTINAL LYMPH. We obtained samples of peripheral lymph draining lengths of intestine in which Peyer's patches were present or absent. NLC were present at 1.7 ± 0.6% and 4.0 ± 2.0%, respectively. Lymph draining a length including a Peyer's patch contains ~10 times more cells than lymph from non-Peyer's patch areas (7). Thus the majority of NLC are derived from the former areas.

Compared to NLC in X.TDL, directly collected NLC show a deficit in NSE 1+ cells (8.4% vs. 30%). Thus, some of the small, less NSE-reactive cells in X.TDL may not be derived from the intestinal wall.

Origin of NLC from a Bone Marrow Precursor. The origin of NLC from chimeric rats [PVG/cXDA → ↯PVG/c] was assessed by autoradiography. Positive cells (eight or more grains) could be distinguished from negative cells (two or fewer grains). Results (Table VII) show that NLC are derived from donor bone marrow.

Discussion

We here describe a method for the collection of large numbers of cells derived from rat peripheral lymph. Sanders and Florey (33) showed that after excision of lymph nodes the original afferent and efferent lymphatics reunite. Removal of the mesenteric lymph nodes in the rat results in the appearance, in thoracic duct lymph, of cells distinct from lymphocytes, which we refer to as nonlymphoid cells. Many of these cells are similar to the veiled cells seen in peripheral lymph of all mammalian species.

However, some small NLC are adherent, show rapid spreading, are actively phagocytic, and are thus probably mononuclear phagocytes. These cells also resemble monocytes cytochemically and are more common in X.TDL than directly collected peripheral lymph. The frequency of these cells in X.TDL is generally very low but increases greatly in infected rats.

Local Origin of NLC. Evidence presented shows that most NLC derive from the intestinal wall. First, similar cells are present in intestinal lymph at a higher frequency than in X.TDL, this difference being explicable by dilution of X.TDL by lymphocytes from nonintestinal sites. Secondly, cells with similar morphology and cytochemistry are found in the lamina propria and in the subepithelial layers of Peyer's patches (34, 35; C. W. Pugh and G. G. MacPherson, unpublished observations). Both NLC and cells in the gut wall bear large amounts of Ia antigen and are bone marrow-derived (36).

Some NLC may derive from the peritoneal cavity. The appearance of NLC-containing carbon in X.TDL following the orthotopic transfer of labeled peritoneal cells might result from inflammation at the cannulation site or physiologically, by migration from the serosal surface into intestinal lymph, a previously unknown route. This interpretation holds whether the donor cells survive or whether the label is acquired by the RPC of the recipient. If the labeled RPC are removed by another mechanism the proportion of NLC derived from the peritoneal cavity would have been underestimated.

Life History of NLC. The constant output of NLC into X.TDL for at least 5 d after cannulation suggests that, unlike the majority of TDL (37), NLC do not recirculate. The [^{3}H]TdR labeling kinetics of NLC are also compatible with rapid turnover and continual replenishment from a dividing precursor pool. Few NLC are produced locally, since 0.5 or 1 h after giving [^{3}H]TdR, no labeled NSE-positive cells are seen in the lamina propria or NLC in X.TDL. Macrophages, Langerhan's cells, and dendritic cells are all bone marrow-derived (38–40). Results presented here show that NLC are bone marrow-derived in radiation chimeras. The nature of the bone-marrow NLC precursor is not known and is under investigation. The in vivo [^{3}H]TdR labeling kinetics of NLC in X.TDL are similar to those described for blood monocytes (41).

Kinetic Behavior of NLC in the Intestinal Wall. The minimum time spent between the last division of the NLC precursor and the appearance of NLC in X.TDL is <24 h. As part of this period will be spent in the blood, the actual minimum transit time through the intestinal wall may be shorter. The first labeled NLC to appear are the smallest NSE 1+ NLC (the monocytoid cells discussed earlier). It is possible that some of these cells may not derive from the intestinal wall. Labeled large, NSE 2+ or 3+ NLC first appear in X.TDL between 24 and 48 h after giving [^{3}H]TdR, suggesting that these cells may also traverse the intestinal wall rather rapidly.

Results from sublethally irradiated animals suggest that only a few NLC take more

than 3–4 d from their final division to their appearance in X.TDL. However, it is possible that irradiation may alter the normal migration of NLC. Therefore unequivocal estimation of the maximum time spent by NLC in the intestinal wall under physiological conditions is not possible. This question could be approached by pulse-chase labeling of NLC in the intestinal wall if a specific NLC marker were available.

These kinetics resemble those of mouse splenic dendritic cells (40). Mouse dendritic cells may be radiosensitive (40), but rat lymph node dendritic cells are clearly radioresistant, irradiation being used in their purification (42).

Classical macrophages are radioresistant. In peritoneum and liver under steady state conditions (38) they turn over more slowly than NLC, but turnover increases in inflammation (43).

Fate of NLC and NLC in Central Lymph. It is generally held that peripheral lymph NLC are filtered out at the first node they encounter and are absent from central lymph (1). However, we have consistently found that small numbers of NLC are present in normal TDL at all stages after cannulation, in contrast to the observations of Roser (44) who, however, was identifying macrophages by their endocytic capacity. Some NLC contain inclusions suggestive of a phagocytic past, but we cannot say whether these cells remain phagocytic. These cells may derive from peripheral lymph and not be filtered out in the nodes or they may enter TDL from other sites. The frequency of NLC in normal TDL is similar to that described by Bell (45) for antigen-laden cells in normal TDL following the intraperitoneal administration of antigen. The relationship between these cell types is under investigation.

In normal animals the majority of NLC must either be retained in the nodes or migrate out via the blood. If they are retained in steady state conditions their life span within the nodes must be relatively short. This aspect of NLC life history is currently under investigation.

Endocytic Activity of NLC. In short-term in vitro assays the majority of NLC are neither capable of phagocytosing large numbers of opsonized or unopsonized particles nor of pinocytosing ferritin. In contrast the monocytoid cells are phagocytic and provide a useful positive internal control. Ferritin was used in these assays because of the potential confusion of using horseradish peroxidase in cells with endogenous activity.

The inability of NLC to take up opsonized particles clearly correlates with the relative lack of Fc and C′ receptors (see below). Lack of uptake of latex suggests that this phagocytic incompetence results from both an absence of specific receptors and a deficit in another part of the internalization pathway.

The mannose receptor (MFR) of macrophages is involved in receptor-mediated pinocytosis of molecules with exposed mannose or fucose residues (13). Macrophages can ingest ferritin, at least partially, in the fluid phase. Therefore, as with phagocytosis, poor pinocytosis by NLC stems from both an absence of receptors and of other components of the system.

In contrast to these in vitro observations many freshly collected NLC contain inclusions that are phago-lysosomal in nature. This suggests that at least some NLC go through a developmental phase, possibly within the intestinal wall, in which they are capable of phagocytosis. Such developmental stages may affect the ability of NLC to handle antigen and perform their putative accessory functions.

Alternatively, antigen processing and presentation may involve the interaction of

different cell types. Contact between phagocytic monocytoid NLC and the larger more NSE-positive NLC was repeatedly observed.

Adherence. Large NLC and macrophages differ in their mode of adherence to glass or plastic. Even when viable NLC are attached to glass with poly-L-lysine they do not spread. This failure is thus not solely due to a defect in adhesion. It may correlate with the absence of endocytic mechanisms. The adhesion of NLC to lymphoid cells (spontaneous rosettes) contrasts with their poor adhesion to glass and plastic and may be of great physiological importance.

Surface Phenotype of NLC. The rosetting assay for FcR activity indicates that some NLC show weak expression of these receptors. However, unlike the FcR of macrophages these NLC FcR are undetectable by "panning." Most NLC lack C'R although sometimes receptor-bearing monocytoid cells were seen.

The presence of low affinity FcR and the absence of C'R on afferent lymph cells from pigs, rabbits, and sheep has been reported (3, 46; I. McConnell, personal communication) and is in agreement with the results presented here for NLC. FcR and C'R are present on Langerhan's cells from guinea pigs (47), but not from other species (4). Langerhan's cells may enter dermal lymphatics (4), but changes in surface phenotype may accompany this migration. All NLC of the appropriate strains express class I antigens recognized by R2/15S and R3/13 (8), products of the rat equivalent of the mouse I-A subregion recognized by MRC Ox 3, 4, and 6 (31), and products of the I-E subregion bound by MRC Ox 17 (31).

Without pure populations of NLC quantitation of antigen expression can only be accomplished by comparison with binding to other cell types or to purified antigen. Rat B lymphocytes express an average of 1.5×10^5 Ia molecules per cell (22). NLC are shown to possess ~10 times as many Ia antigenic determinants (i.e., 1.5×10^6 Ia molecules per cell). Mouse splenic dendritic cells express similar levels (48). This is compatible with the putative role of NLC as accessory cells. Although Ia expression alone does not determine the ability to stimulate an MLR (49, 50) NLC have been shown in this laboratory to be potent stimulators (51) and have also been implicated as passenger leukocytes in renal allograft rejection (52).

The lack of expression by NLC of the W3/25 antigen is important because this antigen is present on macrophages, including those that are Ia positive, in a variety of sites (28, 36) as well as subpopulations of thymocytes and mature T cells (T_H) (23, 27). However the expression of W3/25 antigen at different stages in the differentiation of NLC and mononuclear phagocytes is unknown.

NLC all express the leukocyte common antigen defined by MRC Ox 1. NLC weakly express W3/13 antigen but do not express the other rat T lymphocyte antigens tested. The W3/13 antigen may be passively acquired, synthesized at a low level, or may be a remnant from expression earlier in differentiation. Low levels of cytophilic surface immunoglobulin have been detected on NLC. This may be explained by the weak FcR activity of some NLC. The absence of lymphocyte markers on NLC is useful in separating these cell types (see below).

Density of NLC. Isopycnic centrifugation shows that NLC vary in density although the majority of NLC are less dense than lymphocytes. The functional significance of this variation in density has not been examined. The overall recovery of NLC after density gradient separation showed a slight selective loss of these cells, perhaps due to underestimation of the proportion of weakly NSE-positive NLC that had lost their

typical surface morphology or from weak adherence of NLC to the surfaces encountered during separation despite rigorous precautions.

Purification of NLC. Results from isopycnic separation have allowed single step density gradients to be designed for use in producing populations enriched in NLC. The differential radiation sensitivity of NLC and lymphocytes also allows the production of NLC-enriched populations. We have developed the combination of these approaches to permit the production of cell populations containing ~80% NLC. Our technique has been successfully employed by others (52).

The adherence of rat NLC described in this paper is too weak to permit reliable enrichment of these cells. Similarly, Klinkert et al. (42) found that adherence was not useful for the purification of rat dendritic cells, although it is essential in preparation of mouse dendritic cells (48).

The fluorescence-activated cell sorter (53), panning (12), and rosette depletion (54) are being used by us to purify NLC on the basis of the surface phenotype established in this paper. Positive selection is hampered by the absence of NLC-specific markers and their tendency to form spontaneous rosettes limits the purity attainable. In negative selection procedures the presence of lymphocyte antigens in these rosettes results in a low recovery of NLC, and may result in a selective loss of the functionally most active NLC.

Another approach uses the cell sorter to separate on the basis of the differential labeling intensity of esterase-rich NLC and lymphocytes when incubated with fluorescein diacetate. This is a nonfluorescent, lipid-soluble esterase substrate that is cleaved intracellularly to produce fluorescein that, being polar, is retained by viable cells. This technique is also limited by spontaneous rosette formation (results not shown).

Heterogeneity of NLC. NLC from lymphadenectomized animals are heterogeneous. Typical monocytes or young macrophages are normally very rare but appear in larger numbers in "dirty" animals. Drexhage et al. (3) describe a small subpopulation of nonlymphoid cells as being phagocytic and resembling classical macrophages.

The majority of NLC differ from mononuclear phagocytes but are themselves heterogeneous with respect to cytochemistry, the presence of inclusions (discussed earlier), and some surface markers (W3/13, sIg, FcR), although they all express large amounts of Ia antigens. The significance of heterogeneity in their surface morphology is unclear because of the lability of this characteristic shown by the reversible effects of density gradient separation. The presence of cells with composite surface morphologies (e.g., fine and blunt pseudopodia—see Fig. 2, top right) may represent transition between forms. Such transitions have been demonstrated in cultures of afferent lymph cells from other sites in other species (M. Wilders, personal communication). NSE staining of NLC is heterogeneous and differs from that seen in human monocytes (55) and alveolar macrophages (56), in being both cytoplasmic and fluoride-sensitive.

Heterogeneity of NLC with respect to large granular peroxidase activity and Feulgen staining presumably correlates with the presence of phago-lysosomal inclusions. The Feulgen-positive cytoplasmic material must have been acquired by the NLC. The peroxidase may be acquired or endogenous. Feulgen-positive "macrophages" have been described in the lamina propria of guinea pigs (35). We find such cells more commonly in the subepithelial area of rat Peyer's patches (not shown), where they may be interacting with M cells (57). It is unclear whether the

heterogeneity of rat NLC reflects distinct cell lineages, different maturation stages of a single lineage or differing local origins, e.g., lamina propria or Peyer's patch.

Relationship of NLC to Other Cell Types. The description of NLC in this paper provides circumstantial evidence relating these cells to lymphoid dendritic cells (40, 42, 48, 58), interdigitating cells (59), and Langerhan's cells (60). Similarly, this circumstantial evidence shows differences between NLC and classical macrophages (61) and follicular dendritic cells (62).

However, this analysis must be treated with caution in view of the observed heterogeneity amongst NLC, differences in the species of origin and modes of extraction of the various cell types, and the well-documented variability of expression of surface markers and enzyme activities at different stages in a single cell lineage. The true relationships between these different cell types can only be established by careful analysis of their differentiation in vivo and in vitro.

Summary

Mesenteric lymphadenectomy in rats is followed by union of peripheral and central lymphatics, allowing the collection of intestine-derived peripheral lymph cells via the thoracic duct for several days. These cells include a proportion of nonlymphoid cells (NLC) that show irregular and heterogeneous surface morphology including long pseudopodia and veils. They stain variably for nonspecific esterase and acid phosphatase and are ATPase-positive. Their nuclei are irregular and some contain cytoplasmic inclusions, some of which show peroxidase activity and/or contain DNA. NLC have a range of densitites generally lower than that of lymphocytes. Freshly collected NLC express the leukocyte-common antigen (defined by monoclonal antibody MRC Ox 1) and Ia antigens (I-A and I-E subregion products defined by monoclonal antibodies) but they show a relative lack of other surface markers normally found on rat B or T lymphocytes (W3/13, W3/25, MRC Ox 12 (sIg), MRC Ox 19) or rat macrophages (FcR, C'R, mannose R, W3/25). In general NLC are only weakly adherent to glass or plastic. Although a subpopulation of NLC appear to have had a phagocytic past, freshly collected NLC fail to phagocytose a variety of test particles in vitro. NLC also appear incapable of pinocytosis in vitro. This heterogeneity may represent distinct subpopulations of NLC or different stages in the development of a single cell lineage.

Direct cannulation of mesenteric lacteals shows that the majority of NLC are derived from the small intestine and their precursors appear to be present both in lamina propria and Peyer's patches. Kinetic studies, following irradiation or intravenous tritiated thymidine, show that the majority of NLC turn over rapidly in the intestine with a modal time of 3–5 d. Studies with bone marrow chimeras show that they are derived from a rapidly dividing precursor present in normal bone marrow. NLC occur at very low frequencies in normal thoracic duct lymph at all times following cannulation.

The evidence presented suggests that NLC closely resemble mouse lymphoid dendritic cells. This conclusion is supported by evidence already obtained showing that NLC are potent stimulators of the semi-allogeneic rat primary mixed leukocyte reaction.

In addition to the cells resembling dendritic cells rare monocytoid cells are found in thoracic duct lymph of lymphadenectomized specific pathogen-free rats. The proportion of these cells increases greatly when the animals are conventionally housed.

It seems probable that the physiological function of NLC is to act as accessory cells in the lymph nodes to which they normally drain. Methods for enriching NLC and thus facilitating analysis of their functions are discussed.

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