Brief Definitive Report

ISOLATION AND IMMUNOCYTOCHEMICAL CHARACTERIZATION OF HUMAN BONE MARROW STROMAL MACROPHAGES IN HEMOPOIETIC CLUSTERS

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Erythroid maturation is invariably found in association with a central macrophage (Mϕ) in erythroid islands (1). Mature Mϕ are present in most tissues, including hemopoietic and lymphoid organs, where they have defined roles in phagocytosis and host defense against infection, and other local trophic functions (2). Mϕ can secrete a wide range of biologically active molecules, including hemopoietic growth factors and other growth modulators (3). Their close association with maturing erythroblasts in hemopoietic tissues (1, 4, 5) therefore suggests specialized functions in erythroid regulation, in addition to phagocytosis of extruded nuclei (1).

Although erythroid islands have been observed in marrow, there have been few attempts to analyze the central Mϕ in terms of its frequency and anatomical location, or phenotype and functional properties. In rodent marrow, stromal Mϕ form an extensive network in situ (6) and have recently been isolated and characterized (4). Stromal Mϕ (or marrow histiocytes) may also be present in smears prepared from human marrow, but erythroid islands are disrupted and the central Mϕ are often damaged. Here, we localize human stromal Mϕ in situ, isolate stromal Mϕ within hemopoietic clusters from marrow, and characterize these cytochemically and immunophenotypically in comparison with freshly harvested and cultured monocytes. Our studies indicate that human marrow contains a distinct and accessible population of specialized tissue Mϕ which could be important in hemopoiesis and a range of pathological conditions.

Materials and Methods

Media and Reagents. DMEM (Gibco-Biocult, Paisley, Scotland) was supplemented with 2 mM L-glutamine, and 20 mM Hepes buffer (Gibco-Biocult). FCS (Biological Industries, Kibbutz Haemek, Israel), and autologous human serum were heat inactivated at 56°C for 30 min.

Sources of Marrow. Cell clusters were isolated from human rib specimens resected during thoracic surgery or from routine marrow aspirates. Unless otherwise stated, specimens for

analyses were selected from subjects without marrow pathology, and who had normal peripheral blood hematological and biochemical indices.

Isolation of Stromal Mφ.   Cell clusters were enriched from marrow cell suspensions by low speed centrifugation followed by velocity sedimentation. Bone specimens were split with secateurs and gently lavaged with DMEM, using a Pasteur pipette. To enrich for clusters and to deplete red cells, other single cells, and fat droplets, suspensions were centrifuged three or four times at $50 g$ at $4°C$ for 5 min. The cluster-enriched pellet was gently resuspended in DMEM and a drop was viewed microscopically to assess the ratio of single cells to cell clusters (4). 10^7 cluster-enriched cells were loaded onto DMEM with 30% FCS in an inverted 50-ml plastic syringe barrel, with a glass bead on the outlet port to prevent eddying during collection. After 45 min of sedimentation at unit gravity at $4°C$, 2-ml fractions were collected and cluster-enriched fractions were cytocentrifuged onto glass slides, air dried, and stored at $-20°C$. Cell suspensions were manipulated gently throughout to avoid dispersion of cell clusters.

Marrow Sections.   Cores of rib marrow were obtained with a Jamshidi needle, snap frozen in liquid nitrogen, and 5-μm sections cut on a Leitz Kryostat 1720.

PBMC and Cultured Blood Monocytes.   PBMC from normal donor peripheral blood were harvested using Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden). PBMC in DMEM with 10% autologous serum were cultured on tissue culture coverslips (Thermanox, Miles Laboratories, Naperville, IL), washed after 1 h, and harvested after 10 d culture at $37°C$ in 5% CO_2.

Cytochemistry.   Acid phosphatase, alkaline phosphatase, nonspecific butyrate esterase, myeloperoxidase, and hemosiderin were stained as described (7).

Monoclonal Antibodies.   mAbs towards human leukocyte–associated antigens were obtained from reagents submitted to the Third International Workshop on Leukocyte Typing Antigens (8); from Dako Ltd., High Wycombe, UK; from Ortho Diagnostic Systems, Raritan, NJ; or from one of the authors' laboratories.

Immunocytochemistry.   Cells and sections were labeled by the alkaline phosphatase–anti-alkaline phosphatase (APAAP) method (9). Monocytes in PBMC were distinguished from lymphocytes by their distinctive nuclei and reduced nuclear/cytoplasmic ratio. >400 freshly harvested or cultured monocytes were scored in three separate experiments for each antigen. Isolated stromal Mφ were identified by their nuclear morphology, low nuclear/cytoplasmic ratio, plasma membrane processes, and the presence of >5 attached erythroblasts. 50–100 stromal Mφ were scored in more than three experiments for each antigen.

Results and Discussion

Localization In Situ and Isolation of Stromal Mφ.   Stromal Mφ could be localized in situ with anti-Mφ mAb Y1/82A, which gave discrete and intense labeling of a cell population with long fine processes arborizing in a uniform network extensively distributed throughout marrow (Fig. 1 *a*). To isolate stromal Mφ, cell clusters were

FIGURE 1.   (*a*) Immunocytochemical staining with anti-Mφ mAb Y-1/82A of bone marrow section reveals a network of arborizing stromal Mφ uniformly distributed throughout the marrow interstitium (APAAP stain; hematoxylin counterstain). (*b*) Bone marrow cells depleted of red cells and other single cells are enriched for cell clusters, most of which are erythroid clusters with a central stromal Mφ (*arrows*), (Giemsa). (*c*) Isolated erythroid cluster with intermediate and late normoblasts surrounding a central stromal Mφ (Giemsa). (*d*) Isolated mixed cluster with both myeloid and erythroid cells attached to a central stromal Mφ. A dividing cell (*arrow*) is seen (Giemsa). (*e*) Isolated erythroid clusters from a pathological marrow sample (see text) show intense staining for hemosiderin of stromal Mφ with cellular processes extending between attached erythroblasts (Perl's acid ferrocyanide reaction; counterstain neutral red). (*f*) Immunocytochemical stain with mAb Y-1/82A of isolated erythroid cluster. Both the stromal Mφ cell body and processes (*arrows*) between attached erythroblasts are visible (APAAP stain; haematoxylin counterstain). Bar, 50 μm.
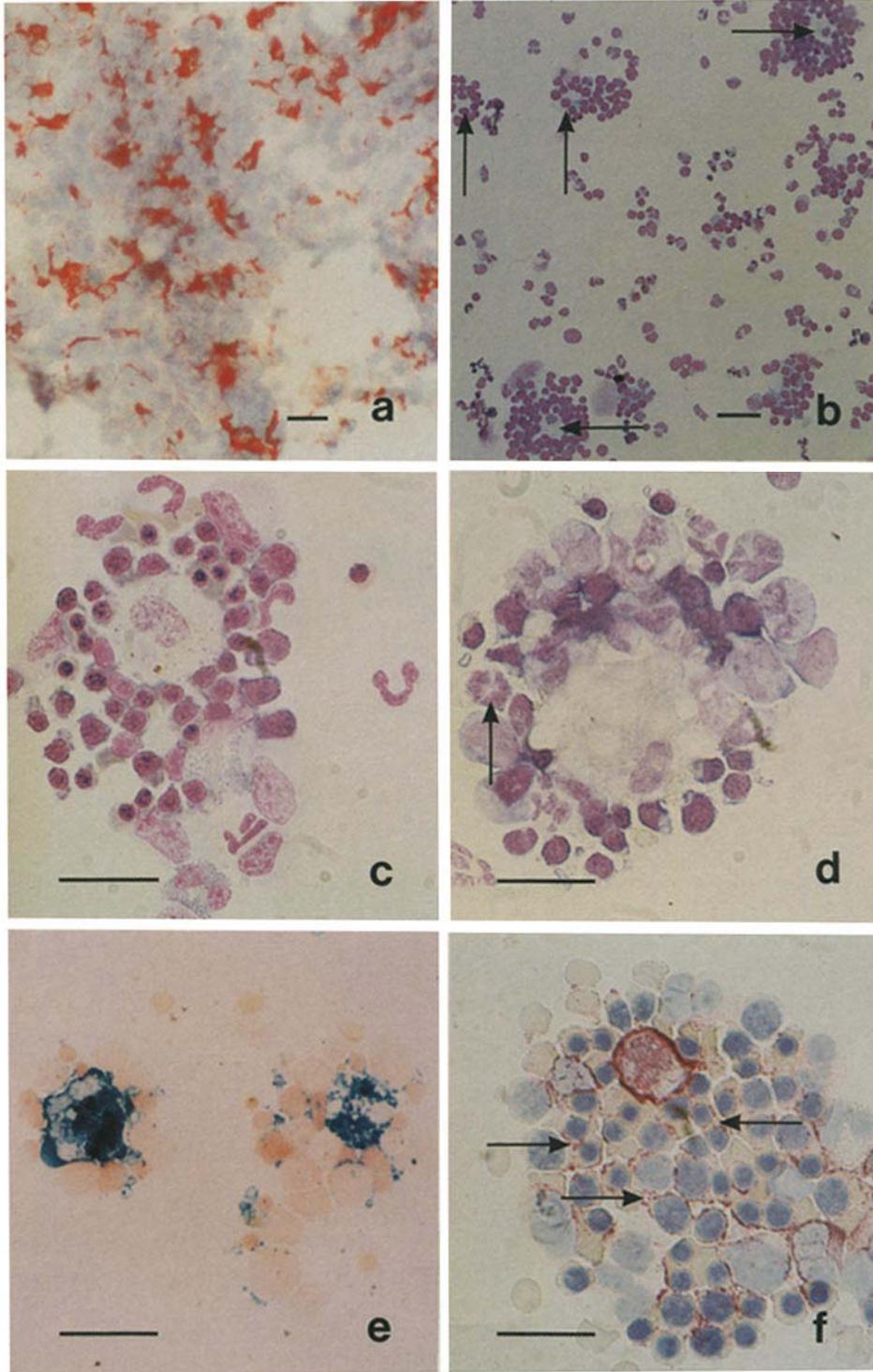
TABLE I

*Yield and Composition of Cell Clusters Harvested from
Human Rib Marrow*

Yields:	
Cell clusters harvested per 10^7 nucleated cells:*	2.2×10^4 $(1-3 \times 10^4)$
Post sedimentation single cell:cluster ratio:*	17 (4-41)
Percent erythroid clusters of all cell clusters:‡	62 (36-96)
Percent erythroid clusters with: 5-10 cells attached:‡	11.9
10-20 cells attached:	40.7
20-30 cells attached:	27.9
>30 cells attached:	19.2
Composition of erythroid clusters:§	
Percent erythroid cells: 73 (52-91)	
myeloid cells: 26 (8-48)	
other cells:‖ 0.5 (0-2.0)	

* Mean (range) of 6 samples of rib marrow.
‡ 500 clusters from six samples were scored for erythroid clusters and cells within clusters were counted.
§ Differential count of cells in 100 erythroid clusters from six samples. Results expressed as mean (range).
‖ Cells of uncertain lineage by light microscopy (Giemsa stain).

enriched ~100-fold by depletion of red cells and other single cells (Fig. 1 *b*, Table I). Clusters obtained from different specimens showed wide ranges in yield and size. The majority of isolated clusters were composed of intermediate or late normoblasts (Fig. 1 *c*), with a variable proportion of myelomonocytic cells, in intimate contact with a central Mφ (Fig. 1 *d*, Table I). Infrequent clusters in which a central Mφ could not be found, consisted of myeloid cells, erythroblasts, or megakaryocytes. Cytochemically, stromal Mφ were positive for acid phosphatase, nonspecific butyrate esterase, and hemosiderin, and negative for alkaline phosphatase and myeloperoxidase. Stromal Mφ isolated from a patient who had received multiple red cell transfusions for refractory anemia showed intense staining for hemosiderin extending along cell processes surrounding erythroblasts (Fig. 1 *e*). Stromal Mφ cytoplasm between erythroblasts could also be identified by acid phosphatase staining (not shown, reference 4) or by immunocytochemical labeling (Fig. 1 *f*, see below). Thus, erythroblasts, and to a lesser extent, myeloid cells, are selectively associated with stromal Mφ, although the relative contributions of specific hemagglutinins characterized on mouse stromal Mφ (4, 5), and other nonphagocytic receptors or adhesive molecules that may specifically bind human hemopoietic cells have not been defined.

Immunophenotype of Stromal Mφ.   Isolated stromal Mφ were strongly positive for several anti-Mφ mAb (Table II, Fig. 1 *f*). Monocytes and megakaryocytes showed weaker labeling and were easily distinguishable as relatively scant, single cells, compared with stromal Mφ. Virtually all stromal Mφ expressed CD4, CD31, HLA-DR, FcRI, FcRII, and FcRIII (Table II). Stromal Mφ also expressed CD11a, CD11c, and CD18 polypeptide chains of the LFA1/Mac-1 family, but showed weak expression of CD11b (Mac-1), as on murine stromal and other tissue Mφ (2, 4). CD35 (CR1) was absent from human stromal Mφ, although attached erythroblasts were strongly positive. Stromal Mφ were also negative for the transferrin receptor epitopes defined by mAb BerT9 and 5E9 (not shown in Table II), contrasting with positively labeled

TABLE II

Antibody*	Antigen‡	Specificity	Blood monocytes		Cultured MØ		Stromal MØ	
			+ §	0–3 + ‖	+	0–3 +	+	0–3 +
			%		%		%	
T9-10¶	CD2	LFA2	0	0	0	0	0	0
T3-10	CD4	—	21	+	40	+	100	2 +
MHM24 (282)	CD11a	α L subunit, LFA1	100	2 +	100	2 +	96	2 +
2LPM19C	CD11b	α M subunit, Mac-1	83	2–3 +	100	2 +	31	+
KB90 (730)	CD11c	α X subunit, p150/95	90	2 +	100	2 +	74	2 +
SJ-1D1 (285)	CD13	—	81	+	100	2 +	52	+
CLB-MON/1 (349)	CD14	—	71	3 +	60	2 +	61	+
MG38 (344)	CD16	FcRIII	5	+	95	2 +	96	1–2 +
MHM23 (283)	CD18	β subunit, CD11a,b,c	100	3 +	100	2 +	84	+
ACT-1	CD25	IL-2 receptor	0	0	3	+	17	+
TM3 (294)	CD31	—	93	2 +	75	2 +	100	3 +
CIKM5 (287)	CD32	FcRII	90	+	100	2 +	100	2 +
TO5 (417)	CD35	C3b receptor	69	2 +	43	2 +	0	0
OKM5	CD36	Thrombospondin receptor	72	+	38	+	31	+
EBM11 (254)	Gp 12	—	91	2 +	100	3 +	94	3 +
Y-1/82A (272)	Gp 12	—	90	2 +	100	3 +	96	3 +
Ki-M8 (303)	Gp 12	—	75	2 +	73	2 +	100	3 +
10.1 (250)	Gp 12	FcRI	ND	—	ND	—	100	2 +
CR3/43	—	HLA-DR	99	2 +	98	1–3 +	100	2 +
Ki-67	—	Proliferation antigen	0	0	<0.2	1–2 +	0	0
BerT9 (837)	—	Transferrin receptor	ND	—	74	+	0	0

* Workshop code numbers and ‡antigen clusters designated by the Third International Workshop on Leucocyte Typing Antigens (reference 8).
§ Percent cells positive; see text for details.
‖ Intensity of staining: 0, absent; +, weak; 2 +, moderate; 3 +, intense.
¶ No staining of stromal MØ was found with IgG1 (T9-10, anti-CD2), IgG2a (35-1, anti-CD2), IgG2b (BW264/56, anti-CD3), IgG3 (Ki-1, anti-CD30), or IgM (9-2, anti-CD2) mouse mAbs to irrelevant epitopes.

erythroblasts. 50–60% of stromal Mφ were positive for CD13 and CD14, and a smaller proportion for CD25 (IL-2 receptor) and CD36 (thrombospondin receptor).

Thus, unlike other human Mφ embedded within tissues, stromal Mφ can be readily harvested for study, allowing direct analysis of their phenotype. In comparison with blood-derived monocytes and cultured Mφ, CD4 expression on stromal Mφ was increased, whereas CD11b, CD35, and transferrin receptor antigen expression were reduced (Table II). CD11a, CD11c, CD14, CD18, CD31, and CD32 were not expressed differently by the three cell populations to a significant degree.

The immunophenotype of the central cell within haemopoietic clusters confirms that these are specialized mature mononuclear phagocytes expressing endocytic receptors (FcR) and HLA-DR. Our current and previous data (4, 5) indicate that stromal Mφ are distinct from monocytes or culture-derived mature Mφ. Further, the Ki-67 proliferation–associated antigen was absent on stromal Mφ, in agreement with thymidine uptake data demonstrating a low mitotic index (<0.1%) for mouse stromal Mφ (4). However, stromal Mφ yields vary widely in several hematological disorders (Lee, S. H., et al., manuscript in preparation), suggesting that stromal

Mφ turnover can be modulated. The absence of transferrin receptor epitopes on human stromal Mφ is of interest since the means by which Mφ accumulate iron stores, and the pathways by which iron may be reutilized by developing erythroblasts are unclear. Human stromal Mφ also express a high and uniform level of CD4, indicating that these cells are potential targets for human immunodeficiency virus, which can be associated with abnormal hemopoiesis.

Further studies are required to elucidate the role of stromal Mφ in hemopoietic cell–cell interactions, as primary sites for infectious organisms, and in storage and secretion in normal and in pathological states.

Summary

Stromal macrophages (Mφ) have been localized in situ and isolated within erythroid clusters from human marrow. Stromal Mφ arborize in an extensive network uniformly distributed throughout marrow interstitium, and express the phenotype CD4$^+$, CD11a$^+$, CD11c$^+$, CD13$^+$, CD14$^+$, CD16$^+$, CD18$^+$, CD31$^+$, CD32$^+$, FcRI$^+$, HLA-DR$^+$, and CD35$^-$, transferrin receptor–negative, and CD11b (weak). They express endocytic receptor antigens, but show significant differences in myeloid antigen expression compared with freshly harvested or cultured monocytes. Human stromal Mφ are therefore specialized mature marrow Mφ that are accessible for further investigations in infectious, storage, or hemopoietic disorders.

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