Hematopoiesis involves simultaneous growth, differentiation, and maturation of several cell lineages (1). In vivo, this process occurs mostly within bone marrow stroma, a specialized microenvironment comprising a fibrous network of reticular cells and an extracellular matrix (2). A similar stroma is needed to sustain hematopoiesis in long-term Dexter-type cultivation experiments (3–5). In both cases the cellular stroma consists predominantly of macrophages (Mφ), fibroblastoid cells, endothelial cells, and adipocytes (6). Many studies have shown intimate associations between stroma and developing hematopoietic cells (7–10) and direct cell-to-cell contacts appear to be necessary for sustained cell growth in vitro (11). Stromal constituents may associate with particular hematopoietic lineages, i.e., fibroblastoid cells with maturing granulocytes and resident Mφ or sinusoidal endothelium with developing erythroid cells (8, 10, 12, 13).

The mononuclear phagocyte has received considerable attention regarding a possible role in regulating hematopoiesis (14). After an appropriate stimulus, Mφ may secrete lineage-specific growth factors (15, 16) and products that potentiate growth, such as interleukin 1 (17). Conversely, they can inhibit cell growth by degrading growth factors (18) and secreting prostaglandins and interferons (19, 20). In addition, regulators of myelopoiesis such as lactoferrin appear to act by altering Mφ function (21). Although these studies have provided valuable information, an important drawback is that the Mφ populations were not isolated from hematopoietic tissues, but mostly from the peritoneal cavity (mouse) or as blood monocytes (human). It is not known to what extent Mφ from these sites are similar in phenotype and/or function to resident bone marrow Mφ (RBMM).

In previous work (10) the latter were characterized by their content of acid phosphatase and uniform distribution in rodent marrow. More recently (22) murine RBMM have been localized immunocytochemically by Mφ-specific surface antigen F4/80 analysis. It was shown that Mφ plasma membrane processes...
Resident bone marrow macrophages ramify throughout the marrow stroma and form clusters with cells of the myeloid as well as erythroid lineages. In the present study we have isolated such hematopoietic clusters by collagenase digestion and characterized their resident Mφ population. Our studies demonstrate that the phenotype of RBMM differs from that of peritoneal Mφ and that resident Mφ in bone marrow stroma associate with proliferating, immature hematopoietic cells, indicative of specialized trophic interactions.

Materials and Methods

Animals. Mice were bred at the Sir William Dunn School of Pathology, University of Oxford and both sexes used between 8 and 12 wk of age. C57BL/6 (H-2b) mice were used unless indicated.

Media and Reagents. RPMI 1640 was obtained from Gibco-Biocult Ltd., Paisley, Scotland. Fetal bovine serum (FBS) was obtained from Serlab UK Ltd., CrawleyDown, Sussex, England and routinely heat inactivated for 30 min at 56°C. Media were supplemented with glutamine (2 mM) and gentamicin (20 μg/ml). Phosphate-buffered saline (PBS) without calcium or magnesium was obtained from Oxoid Ltd., Basingstoke, England. Collagenase was purchased from Boehringer Corp., Lewes, East Sussex, England and DNase (type 1) from Sigma Chemicals Ltd., Poole, Dorset, England. Lung-conditioned medium (lung CM) was prepared according to Sheridan and Metcalf (23). [3H]thymidine (2Ci/mmol) ([3H]TdR) was obtained from Amersham International PLC, Amersham, Bucks, England.

Antibodies. The following monoclonal antibodies (Ab) were obtained as shown and used as concentrated supernatants at saturation: F4/80, a rat Ab specific for mature mouse Mφ (24); M1/70 (25), which binds to the iC3b receptor (CR3) on mouse neutrophils, peritoneal Mφ, and natural killer (NK) cells (26) (Dr. T. Springer, Harvard Medical School, Boston MA); 7/4, a rat Ab directed to a polymorphic antigen on mouse neutrophils (27) and immunologically activated peritoneal Mφ (28); M5/114, a rat anti-mouse Ab that recognizes Ia antigens (I-Ak,K-Ek) (29) (Dr. H. Waldmann, Department of Pathology, University of Cambridge); and 2.4G2, a rat Ab directed to the trypsin-resistant Fc receptor (FcR) for IgG1/2b isotypes (30) (Dr. J. Unkeless, The Rockefeller University, New York). Monoclonal mouse anti-sheep erythrocyte (E) Ab U88 (IgG2b) and UM-2 (IgG2a) were from Dr. B. Diamond (Albert Einstein College of Medicine, Bronx, NY) (31). An IgM fraction of rabbit anti-E antiserum was a gift of Dr. R. Sim, University of Oxford.

Bone Marrow Cells. Cellular clusters with RBMM were obtained by enzymatic digestion of femoral marrow plugs. Mice were killed by cervical dislocation, both femurs excised, and the epiphyses removed. The plugs were extruded by inserting a 23 gauge needle into one of the cut ends and flushing gently with an RPMI solution containing 0.05% collagenase and 0.001% DNase. The tissue from two femurs was suspended in 10 ml of the same enzyme solution and digested at 37°C by tumbling at one revolution per second for 1 h. Digestion was stopped by adding FBS to a final concentration of 1% vol/vol. At this stage the marrow plug fragments were no longer visible and had been dispersed to yield a homogeneous suspension.

Enrichment of Bone Marrow Clusters. For phenotypic and functional analyses, clusters were separated from single cells by unit gravity velocity sedimentation in RPMI containing 30% FBS. In some experiments a crude separation was achieved by layering 5 ml of tissue digest over 10 ml RPMI plus 30% FBS in a 50 ml Falcon centrifuge tube (Becton Dickinson & Co., Oxnard, CA). After 1 h at room temperature, 14 ml of medium were carefully aspirated; the remaining 1 ml contained >90% of clusters. In other experiments, digests from two mice were pooled and centrifuged at 100 g for 10 min. The cells were suspended gently in 3 ml RPMI, layered over 20 ml RPMI plus 30% FBS and, in turn, over a 3 ml cushion of Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) in a 20 ml syringe. The syringe was connected to rubber tubing that was clamped off. After
60 min at room temperature, 2-ml fractions were collected and inspected by phase contrast microscopy for the presence of clusters. Fractions containing clusters free of contaminating single cells were pooled and washed twice in RPMI by centrifugation at 100 g, 10 min. To obtain column fractions with differing proportions of single cells and clusters for analysis of TdR incorporation and progenitor content (see below), the same procedure was used except that the sedimentation time was shortened to 30 min. To obtain sufficient numbers of adherent RBMM for phenotypic analysis, purified clusters from six mice (three columns) were suspended in 2.4 ml RPMI plus 10% FBS and 100-μl aliquots added as "bubbles" to precleaned 11-mm diam glass coverslips in a 24 well culture dish. After 30 min incubation at 37°C, wells were gently flooded with 1 ml RPMI plus 10% FBS and left to incubate for a further 3 h. These and all subsequent incubations of cells at 37°C were in the presence of 5% CO₂. To remove cluster nonadherent cells while retaining adherent Mφ, coverslips were rinsed five times with PBS and left in PBS for 30 min at room temperature. Clustering cells were then detached by repeated and direct gentle flushing with PBS. This resulted in a population of adherent cells with the characteristic morphology of RBMM, contaminated with up to 50% monocytes and neutrophils.

Characterization of Clusters and Cells in Clusters. Clusters were purified as described above, without centrifugation. Fractions containing "pure" clusters were pooled and adjusted to 10⁶ clusters per ml, and cytospin smears were prepared from 0.5-ml aliquots. For immunocytochemical analysis clusters were fixed wet in 0.125% glutaraldehyde in PBS; for morphology, smears were air dried, fixed in methanol, and stained with Giemsa. For single-cell analysis pure clusters or unfractionated cell suspensions were washed three times in PBS by centrifugation at 100 g, 10 rain. The cells were suspended at 2 x 10⁵/ml in PBS containing 0.5 mM EDTA and 5 mM glucose, left to chill on ice for 30 min, and dispersed into a single-cell suspension by repeated passage through a 27 gauge needle. Cytocentrifuge preparations were made and fixed as described above.

Peritoneal Mφ. Adherent resident peritoneal Mφ (RPM) were obtained by lavage with PBS. In some experiments they were treated after isolation with collagenase and DNase, as for bone marrow.

Immunocytochemistry. The presence of various cell surface antigens was assessed by immunoperoxidase labeling using an avidin-biotin detection system (Vectastain; Seralab UK Ltd.). Cells on coverslips were fixed for 10 min at room temperature in 0.25% glutaraldehyde in PBS and washed three times in PBS, and unreacted aldehyde groups were quenched by incubation in PBS containing 10% FBS, for 1 h at room temperature. Coverslips were then rinsed three times with PBS and endogenous peroxidase activity destroyed by incubation in methanol with 0.3% H₂O₂. In the case of M1/70 this treatment was found to destroy antigenic activity on RPM and was therefore carried out after incubation with M1/70. Coverslips were rinsed three times in PBS and stained as described (32). In all experiments, controls without a first Ab or with an irrelevant first Ab were included. A negative strain control was also used when staining with M5/114 or 7/4.

Cytochemistry. Unless stated otherwise all substrates and reagents were purchased from Sigma Chemicals Ltd. To demonstrate acid phosphatase activity, we used the method of Burstone (33), with Naphthol AS-B1 phosphate, pararosaniline. The method of Ornstein (34) was used, with α-naphthyl butyrate as substrate, to demonstrate nonspecific esterase type I. The presence of granular peroxidase was tested as described by Kaplow (35), using diaminobenzidine hydrochloride as substrate. The method of Cordell et al. (36) was used to detect alkaline phosphatase activity, using naphthol AS MX phosphate and fast red TR salt.

Complement Receptors. RBMM or collagenase-treated or untreated RPM on coverslips were placed in 0.5 ml RPMI in wells. To these was added 0.1 ml of a 5% vol/vol suspension of E, coated sequentially with a subagglutinating concentration of rabbit IgM anti-E Ab and fresh C5-deficient DBA/2 mouse serum. Controls received IgM coated E or E alone. After 30 min at 37°C nonattached E were removed and the Mφ were fixed in 0.25% glutaraldehyde in PBS and examined by phase contrast microscopy for the presence of
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attached or ingested E. Ingestion was assessed after lysing bound E with NH₄Cl before fixation.

**Fc Receptors.** Mϕ on coverslips were placed in 0.5 ml RPMI in wells. To these was added 0.1 ml of a 5% suspension of E coated with a subagglutinating amount of mouse monoclonal anti-E Ab. After 60 min at 37°C, nonattached E were removed and the Mϕ were fixed in 0.25% glutaraldehyde in PBS and examined by phase contrast microscopy for the presence of attached or ingested E.

**Mannosyl/Fucosyl Receptors (MFR).** The presence of MFR was demonstrated by mannan-inhibitable binding of a ¹²⁵I trace-labeled glucoconjugate of mannose–bovine serum albumin (mannose-BSA) (37). Single-cell analysis was carried out by autoradiography (38).

**Respiratory Burst.** Reduction of nitroblue tetrazolium (NBT) to an insoluble blue formazan deposit was used to detect respiratory burst activity. Mϕ on coverslips were placed in 0.5 ml RPMI containing 1 mg/ml NBT (Sigma Chemicals Ltd.). To these was added 5 μg of boiled zymosan (Sigma Chemicals Ltd.) in RPMI. After 30 min at 37°C, coverslips were rinsed three times in RPMI and fixed in 0.25% glutaraldehyde. Coverslips were examined by phase contrast microscopy for ingestion of zymosan and by bright-field microscopy for deposition of formazan. Controls without zymosan were included.

**[³H]Tdr Incorporation.** (a) Single-cell analysis: Bone marrow cell suspensions were enriched for clusters by a one-step separation as described above. 10⁶ clusters in 1 ml RPMI plus 10% FBS were added to coverslips in wells. 1 μCi of [³H]Tdr was added to each well and the cells incubated for 2 or 16 h at 37°C. Coverslips were gently rinsed three times by immersion in RPMI and incubated for 1 h in RPMI plus 10% FBS. Coverslips were then rinsed three more times and fixed in 0.25% glutaraldehyde in PBS. To determine whether RBMM incorporated [³H]Tdr under these conditions, some coverslips were treated to remove clustering cells (see above) before fixation. RBMM were routinely stained immunocytochemically with F4/80 before processing for autoradiography. Coverslips were exposed to Ilford K5 emulsion (Ilford Ltd., Basildon, Essex) for 2 wk and then developed.

(b) Quantitative Analysis. Fractionated bone marrow cell suspensions were assessed for their content of clusters and single cells, washed twice in PBS, and made into a single-cell suspension by repeated passage through a 27 gauge needle. The cells were adjusted to a concentration of 10⁶ nucleated cells/ml in RPMI and 10% FBS. 50- or 100-μl aliquots of cell suspension were incubated in 96-well flat-bottomed microtiter plates (Linbro; Flow Laboratories, Inc., McLean, VA). The volume of each well was made up to 180 μl with RPMI plus 10% FBS, and 1 μCi of [³H]Tdr in 20 μl RPMI plus 10% FBS was added. Lung CM was added to some wells to give a final concentration of 15% vol/vol. After incubation for 16 h at 37°C in 5% CO₂ in a fully humidified incubator, [³H]Tdr incorporation into DNA was assessed using a cell harvester (ILACON Ltd., Tonbridge, Kent, UK), followed by scintillation counting. In these experiments the percentage of cells in clusters for each fraction was derived from the following equation: 100% — [(number of single cells before dispersion/number of single cells after dispersion) × 100].

**Granulocyte/Macrophage Colony-forming Cell (GM-CFC) Assay.** The number of GM-CFC in fractionated bone marrow cell suspensions was determined as described (23). Care was taken to ensure that clusters were completely dispersed into single-cell suspensions. Cloning medium contained 10–20% lung CM as a source of GM colony-stimulating factor (GM-CSF), 20% heat-inactivated (56°C, 30 min) FBS, 20% Bactoagar (Difco Laboratories, Inc., Detroit, MI; stock solution 1.65% wt/vol in distilled water), 20% double strength RPMI, and 20–30% RPMI. Colonies (>50 cells) were scored at day 7 using a low power inverted microscope. Colony morphology was determined by staining with Giemsa after agar plugs had been dried onto glass slides and fixed with methanol.

**In Vitro Culture of Clusters.** Purified clusters at 10⁵ to 10⁶ per coverslip were cultivated at 37°C in 5% CO₂ in 1 ml RPMI containing 10% FBS. At various times up to 72 h, coverslips were rinsed gently in RPMI, fixed, and stained for immunocytochemical or morphological analysis, as described above.
Results

**Isolation and Characterization of Bone Marrow Clusters.** To isolate RBMM from the marrow stroma we adopted a strategy of gentle enzymatic dispersion. Techniques based on physical dispersion were avoided in view of the delicate Mø processes previously observed in situ (22). Indeed, after preparing single-cell suspensions by conventional methods (vigorous pipetting or passage through fine-gauge needles), we were unable to detect adherent cells with the morphology of mature Mø. We tested various batches of collagenase from different commercial suppliers and found that Boehringer collagenase was the most efficient at dispersion in the absence of detectable toxic effects. DNase was normally found to be a necessary additive; in its absence, gelatinous aggregates sometimes formed during digestion. 60 min of digestion resulted in optimal tissue dispersion and periods up to 2 h did not appear to affect the composition of the digested material. After digestion with collagenase the majority of marrow plug fragments had been dispersed into an homogeneous suspension of cells. We routinely recovered $2.5 \pm 0.9 \times 10^7$ nucleated cells per two femora, most in the form of single cells, but including a small number of clusters (more than five cells) at a frequency of $\sim 2.2 \times 10^2$ per $10^5$ nucleated cells (Table I). Clusters were extremely variable in size, containing 5–100 cells with an average of 35 per cluster.

To analyze the cell types contained within clusters, we first purified them by velocity sedimentation on columns of 30% FBS. Centrifugation was avoided during all stages of purification to minimize the possibility of artifactual cluster formation; Mø were detected by immunocytochemistry with the Mø-specific Ab, F4/80. Intense F4/80 staining was found in 70% of all clusters (Table II), but staining was undetectable in the single-cell population. In general, two patterns of staining were observed depending on the size of cluster. In $\sim 50\%$ of small clusters (5–10 cells, 40% of total), a single F4/80+ cell formed the center of classic erythroblastic islets (12, 13), the majority of which contained immature neutrophils as well as nucleated red cells (Table II and Fig. 1A). The small clusters that lacked staining for F4/80 were largely composed of aggregates of mature neutrophils. Of the larger clusters (>10 cells, 60% of total), >80% were

| Table I | Yield of Nucleated Cells, Clusters, and Resident Bone Marrow Mø from Collagenase-digested Bone Marrow |
|-----------------|---------------------------------------------------------|
| Total nucleated cells | $2.5 \pm 0.9 \times 10^7$* |
| Total clusters (>5 cells) | $5.4 \pm 1.6 \times 10^4$* |
| Percent clusters of 5–10 cells | 40% |
| Percent clusters of >10 cells | 60% |
| Average number of cells per cluster | 35 (range, 5–100) |
| Percent of total cells in clusters | 7 ± 3%* |
| Percent clusters glass-adherent | >90% |
| Approximate number of RBMM | $2.5 \times 10^5$4 |

Data obtained with 3-mo-old female C57BL/6 mice.

* ±1 SD; recovery from two femora. Data derived from >10 independent experiments.

4 Estimate for two femora, based on 0.9% frequency of large, strongly F4/80+ Mø (see Table III).
Analysis of Intact Bone Marrow Clusters for Different Cell Types

| Cell type         | Percent of clusters containing the different cell types | 5-10-cell clusters | >10-cell clusters | Total clusters* |
|-------------------|--------------------------------------------------------|--------------------|------------------|-----------------|
|                   | %                                                      | %                  | %                |                 |
| Resident Mφ*      | 53                                                     | 85                 | 71               |                 |
| Myeloid†          | 93                                                     | 85                 | 89               |                 |
| Erythroid‡        | 61                                                     | 22                 | 40               |                 |
| Myeloid and Erythroid | 54                                                   | 10                 | 30               |                 |
| Fibroblastoid§     | 17                                                     | 34                 | 26               |                 |

Purified clusters were cytocentrifuged onto glass slides and processed for immunocytochemistry or morphological analysis. Results are for duplicate slides; 100 clusters were analyzed per slide.

* 40% of clusters contained 5–10 cells; 60% of clusters contained >10 cells.
† Based on intense F4/80 staining.
‡ Based on morphology and F7/4 staining.
§ Based on morphology.
§ Based on alkaline phosphatase staining.

Stained strongly with F4/80. The pattern of staining suggested that Mφ membrane processes ramified extensively, establishing intimate contact with hematopoietic cells distal from the Mφ cell body (Fig. 1B). Frequently, the larger clusters contained more than one strongly staining cell. The larger clusters were predominantly myeloid with only ~20% containing erythroid cells, compared with 60% of small clusters (Table II). In addition, ~30% of the larger clusters and 20% of the smaller clusters showed reactivity for alkaline phosphatase, previously shown to be a useful marker for murine fibroblastoid reticulum cells (10). Similar to F4/80 staining, alkaline phosphatase reactivity was found diffusely within clusters (Fig. 1C).

To analyze the cell types within clusters more closely, purified clusters were dissociated into single-cell suspensions. To avoid damaging the Mφ membrane processes during dissociation, the clusters were incubated for 30 min in ice-cold PBS containing 0.5 mM EDTA to promote retraction of the processes, and single-cell suspensions were prepared by gentle passage through a fine-gauge needle. This population was compared with unpurified suspensions of bone marrow digest treated identically (Table III). As expected, large, intensely F4/80-staining cells were enriched fivefold after purification. These cells possessed delicate plasma membrane processes (Fig. 1, D and E), had a nuclear/cytoplasmic...
### TABLE III

**Characterization of Cell Types in Bone Marrow, Before and After Purification of Clusters**

| Cell type              | Pre purification | Post purification | Fold enrichment |
|------------------------|------------------|-------------------|-----------------|
| Resident Mϕ*           | 0.9              | 4.4               | 5               |
| Immature monocytic†    | 9.2              | 13.4              | 1.5             |
| Neutrophil‡            | 26.0             | 5.8               | 0.2             |
| Immature myeloid†      | 19.8             | 34.6              | 1.8             |
| Erythrocyte            | 12.2             | 4.4               | 0.4             |
| Immature erythroid†    | 7.5              | 11.4              | 1.6             |
| Fibroblastoid†         | 0.6              | 2.0               | 3.3             |
| Undifferentiated†      | 24.0             | 24.0              | 1.0             |

Unfractionated cell suspensions or purified clusters were dissociated into single-cell suspensions and cytocentrifuged onto glass slides. Results of duplicate slides; 300 cells (nucleated + enucleated) scored per slide.

* Based on intense F4/80+ stain.
† Based on morphology/weak F4/80 staining (see text for details).
‡ Based on morphology.
§ Based on alkaline phosphatase reactivity.
¶ Includes lymphocytes and blasts.

The foregoing results demonstrate that clusters contained two populations of F4/80-staining cells, which could be differentiated on the basis of size, intensity of staining, and morphology. Unlike the large, arborized, strongly F4/80+ cells, weakly stained cells were comparatively small and rounded, with no plasma membrane extensions. They resembled promonocytes and monocytes, with reniform nuclei and a nuclear/cytoplasmic ratio >1.

In conclusion, clusters isolated after collagenase treatment were very similar in composition to those described previously in vivo (22) and are therefore unlikely to be artifacts of the digestion procedure. They contain one or more central stromal Mϕ and other reticulum cells that selectively associate with immature, terminally differentiating erythroid and myelomonocytic cells.

**Isolation of RBMM.** To see whether RBMM in clusters could be isolated through attachment to glass coverslips, purified clusters were washed and gently resuspended in RPMI plus 10% FBS. By 2–3 h of incubation at 37°C, >90% of clusters had become firmly adherent. Careful inspection revealed that most of these were underlaid by rapidly spreading cells, the outlines of adherent plasma membrane being clearly visible (Fig. 2A). The majority of clustering cells could
FIGURE 2. Phase contrast micrographs of bone marrow clusters and RBMM after adhesion to glass coverslips. (A) Intact cluster showing spreading of MØ plasma membrane underlying attached hematopoietic cells. (B) Partially stripped clusters revealing aggregates of underlying RBMM. (C) Fully stripped clusters. (D) Higher power of RBMM showing phagocytic inclusions and delicate plasma membrane processes. (E) Two RBMM with a contaminating non-MØ adherent cell (arrow) which displays binuclearity and densely granular cytoplasm, features that are absent from RBMM. Bar, 10 μm.

be removed at this stage by gentle direct flushing of coverslips. This left a population of extensively spread cells, the upper surfaces of which were attached to variable numbers of small, refractile cells (Fig. 2B). To completely strip the underlying spread cell, it was necessary to incubate the coverslips in calcium- and magnesium-free PBS for 15-30 min, followed by gentle, direct flushing with a wide-bore pipette. Vigorous pipetting led to a poor recovery of adherent cells, together with signs of extensive plasma membrane damage.

By phase contrast microscopy, >90% of the well-spread cells revealed by this method possessed morphological features of mature MØ, including oval nuclei, perinuclear pinocytic vesicles, phase-dense lysosomes, long filamentous mitochondria, and variable amounts of phagocytic inclusions (Fig. 2, C and D). Some of these well-spread cells possessed elaborate branched processes that, in partially stripped clusters, appeared to cradle attached hematopoietic cells (Fig. 1E). The extensive spreading and process formation was not a consequence of treatment with collagenase. The few RBMM that could be obtained in its absence (without
mechanical disruption) showed identical morphology. Moreover, RPM treated
similarly with collagenase and DNase showed no morphological differences from
conventionally prepared cells. In addition to RBMM, a variety of other adherent
cells were present, usually making up ~50% of the total. Most of these were
small monocytes and immature neutrophils, but occasionally we observed large
well-spread cells resembling osteoclasts, with multiple nuclei and densely granular
cytoplasm (Fig. 2E). Cells resembling Steinman-Cohn dendritic cells (39) were
not observed.

RBMM were also isolated from other mouse strains, including CBA T6T6,
BALB/c, and ASN. For all strains, regardless of sex, there were no significant
differences in either yield or morphological appearance of clusters and RBMM.
Counts of coverslips indicated that the maximal recovery of adherent, well-
spread F4/80+ RBMM was ~10^5 per two femora of a 3-mo-old mouse (see Table
I). Larger yields of RBMM could be obtained by more prolonged incubation
before stripping (up to 12 h) but this was accompanied by increased contamina-
tion by adherent fibroblasts and monocytes contained within clusters (see below).
We therefore routinely stripped clusters after 3 h of adherence for phenotypic
analysis. Finally, the age of mice did not greatly influence RBMM yields. In
general, recovery of RBMM was proportional to the number of femoral marrow
cells in the starting population.

**Phenotype of RBMM.** To verify that the well-spread cells described above were
mononuclear phagocytes, we analyzed this population for the presence of various
cell surface antigens and receptors, as well as for histochemical markers normally
associated with Mφ (Table IV). The resulting phenotype was compared with that
obtained for collagenase-treated or untreated RPM. The bi- and multinucleate
cells and small adherent monocytes and neutrophils were readily distinguished
by size and morphology from RBMM and were not included in this analysis
except where they served as a useful internal control. Several interesting features
emerged. Both resident Mφ populations stained uniformly with F4/80 and 2.4G2
(IgG 1/2b FcR), although the intensity of staining for both antigens was consid-
erably greater with RBMM. In contrast to RPM, however, RBMM had no
detectable M1/70 (Mac-1) antigen, thought to be the ligand-binding site of CR3
(26). The absence of this antigen was also demonstrated on RBMM from mouse
strains BALB/c, ASN, and CBA T6T6. To verify that RBMM lacked Mac-1, we
carried out rosetting assays for CR3 and for C3b receptors (CR1) by using
complement-coated E. However, a difficulty encountered during these assays
was the presence of very high background rosetting, to 50–90% of RBMM with
the IgM-coated E or unopsonized E used as controls. Regardless of complement
opsonization, there was no change in either the percentage of RBMM forming
rosettes or in the number of E bound or ingested per Mφ, suggesting that
complement receptors were absent. In contrast, complement receptors were
readily demonstrable on the contaminating monocytes and neutrophils (Fig. 3A)
as well as on normal or collagenase-treated RPM, which did not rosette with
IgM-coated or unopsonized E. A detailed account of the "E receptor" will appear
in a forthcoming publication.

To determine the proportion of RBMM that expressed Ia antigens, we used
monoclonal Ab M5/114, which detects I-A-encoded Ia molecules on mouse
Comparison of Phenotype of Resident Bone Marrow and Peritoneal Mφ from C57BL/6 Mice

| No. of Exp. | RBMM                               | RPM                               |
|------------|------------------------------------|-----------------------------------|
| Surface antigens                                      |                                    |                                   |
| F4/80      | >20                                | +++*                             | ++                                 |
| M1/70 (Mac-1, CR3)                                   | 6                                  | ~                                | ++                                 |
| 2.4G2 (FcR IgG1/2b)                                  | 2                                  | +++                             | ++                                 |
| M5/114 (Ia)                                        | 6                                  | ++ (20–60%)                     | + (5–20%)                          |
| 7/4        | 4                                  | ~                                | ~                                  |

| Surface receptors                                     |                                    |                                   |
| Zymosan                                             | 3                                  | +++                             | ++                                 |
| Phagocytosis                                         | 2                                  | ~                                | +/−                                |
| Respiratory burst                                    | 3                                  | +++ (80–100%)                   | + (25–40%)                         |
| FcR IgG2a†                                           | 3                                  | +++                             | ++                                 |
| FcR IgG2b†                                           | 2                                  | ++                              | ++                                 |
| MFR (mannan-inhibitable)                             | 2                                  | ++                              | ++                                 |
| Complement‡                                          | 3                                  | ~                                | ++                                 |
| Sheep erythrocytes‡                                   | >20                                | +++ (50–90%)                    | −                                  |

| Histochemistry                                       |                                    |                                   |
| Acid phosphatase                                     | 2                                  | ++                              | ++                                 |
| Non-specific esterase 1                               | 2                                  | ++                              | ++                                 |
| Peroxidase                                           | 2                                  | ~                                | −                                  |
| ATPase                                               | 1                                  | ++                              | ++                                 |
| Alkaline phosphatase                                  | 4                                  | ~                                | −                                  |

* >95% of cells were positive unless otherwise indicated. (−) Negative; (+) weak; (++) moderate; (+++) strong.
† Score on ingestion, ≥1 E.
‡ See text.
§ Score on binding, >4 E.

strains of haplotype b, d, and q, and I-E-encoded Ia molecules on haplotypes d and k (29). Thus, using C57BL/6 (H-2b), CBA T6T6 (H-2k) and BALB/c (H-2d) mice, we were able to study the expression of I-A-, I-E-, and I-A plus I-E-encoded molecules, respectively. For all strains, we observed variation in staining for individual mice, but a general pattern emerged. Positive RBMM staining was found on 20–60% of C57BL/6 (I-A), 0–5% of CBA T6T6 (I-E), and 60–80% of BALB/c (I-A plus I-E). No staining was found on RBMM from the negative strain control, ASN (H-2s). There was marked heterogeneity in the intensity of staining on individual Mφ. Regardless of mouse strain, ~50% of the positive subpopulation showed intense deposition of diaminobenzidine (DAB) reaction product; the remainder stained faintly (Fig. 3C). There were no apparent differences, morphological or otherwise, between the intensely positive, weak, or negative subpopulations. In contrast to the results obtained with RBMM, <20% of RPM bore Ia antigens, irrespective of strain.

Quantitative difference between RBMM and RPM were also observed for the expression of two distinct FcR, which bind IgG2a and IgG2b isotypes, respectively (31). To overcome the problem of background rosetting with unopsonized E used as controls, Mφ were scored for ingestion; it occurred in <20% of RBMM in the absence of opsonization. Virtually all RBMM were avidly phagocytic for
E coated with either Ab, whereas binding and/or ingestion were considerably less for RPM (Table IV). In this regard, RBMM resemble inflammatory peritoneal Mϕ, which have previously been shown to express high levels of both FcR (40).

Another noteworthy feature of RBMM was their inability to express a respiratory burst in response to unopsonized zymosan, known to be a potent stimulator of this function in inflammatory or activated Mϕ. Contaminating monocytes and neutrophils in these preparations were clearly triggered (Fig. 3B), demonstrating that collagenase treatment was not the cause of the Mϕ unresponsiveness. Moreover, large numbers of zymosan particles were ingested by RBMM, confirming the phagocytic nature of these cells. RPM were weakly triggered but showed detectable NBT reduction after uptake of zymosan.

Other characteristics of RBMM were similar to those of RPM. These included the presence of mannan-inhibitable receptors for mannosyl/fucosyl-terminated glycoconjugates (Fig. 3D) and the enzymes ATPase, nonspecific esterase type 1, and acid phosphatase (not shown). Staining for the latter was particularly intense around the phagocytic inclusions. The absence of alkaline phosphatase reactivity in RBMM distinguished them from stromal fibroblastoid cells, which rarely attached to coverslips under the conditions used (see above).

**Growth Properties of Cells Forming Clusters with RBMM.** In view of the phagocytic nature of RBMM observed in vivo and in vitro, it was important to determine whether clustering cells at the Mϕ surface were in the process of dying and being ingested or, alternatively, were viable and proliferating actively. This would be an important first step in defining the function of RBMM in hematopoiesis. Two approaches were taken to examine the growth properties of clustering cells: (a) quantitative and qualitative analysis of [3H]TdR incorporation to assess the growth of clustering cells in comparison with nonclustering cells, with and without exogenous growth factors, and (b) enumeration of myelomonocytic progenitor cells (GM-CFC) in the two populations. Lung CM was used as a source of GM-CSF (23). In initial experiments, clusters were purified 8–10-fold by one-step separation over FBS and compared with the depleted fraction. In three independent experiments, the enriched fractions incorporated 30,200 ± 1,560 cpm per 10^5 nucleated cells whereas the depleted population incorporated 8,560 ± 1,270 cpm per 10^5 cells. In the same experiments, we assayed for the progenitor content and found that the cluster-enriched population contained 89 ± 13 CFC per 10^5 nucleated cells compared with 190 ± 19 in the depleted fraction. Examination of culture dishes at the onset of the colony formation assays confirmed that the clusters were completely dispersed into single-cell suspensions.

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**Figure 3.** Characterization of RBMM. Phase contrast micrograph (A), bright field (B–E). (A) Rosetting of complement-coated E to contaminating neutrophils and monocytes (small arrows), but not to RBMM (large arrows). (B) Absence of RBMM respiratory burst activity in response to zymosan, despite ingestion (small arrows). Formazan deposition is evident around contaminating neutrophils and monocytes with zymosan (large arrows). (C) Immunocytochemistry showing heterogeneity of Ia expression on RBMM. Negative (*), weak (**), or strongly staining (***') cells are present. (D) Autoradiographic demonstration of MFR expression on RBMM, also stained immunocytochemically with Ab F4/80. Grains overlying RBMM reflect binding of [125I]mannose/BSA in the absence of mannan. (E) Autoradiograph showing [3H]-TdR incorporation into clustering cells, pulsed in vitro for 2 h. Underlying RBMM stained immunocytochemically with Ab F4/80. Bar, 10 μm.
Figure 4. Correlation of $[^{3}H]$Tdr incorporation with the proportion of cells in clusters. A digested bone marrow cell suspension from six femurs was fractionated by velocity sedimentation in 30% FBS for 30 min at room temp. Each fraction was analyzed for the number of clusters and single cells (top; note different scales). Cells were washed and dispersed into single-cell suspensions and $5 \times 10^9$ cells from each fraction cultivated for 16 h with 1 $\mu$Ci of $[^{3}H]$-Tdr without exogenous growth factor. Results (bottom) show mean incorporation of $[^{3}H]$Tdr of quadruplicate cultures for each fraction. Replicates varied <10% of mean values; standard deviations not shown. $[^{3}H]$Tdr incorporation is compared with the percentage of cells originally in clusters for each fraction (C). (For calculation, see Materials and Methods). Addition of lung CM (15% vol/vol) doubled the $[^{3}H]$Tdr incorporation in all fractions (not shown). Similar results were obtained in two independent experiments.

After 7 d of culture there were no apparent differences in the sizes of individual colonies formed in the two groups. However, microscopic examination of cells from colonies revealed a higher frequency of mixed (Mφ and granulocyte) colonies in cluster-enriched fractions. In contrast, colonies derived from cluster-depleted fractions were largely granulocytic (data not shown).

As indicated by previous studies (41), the higher frequency of mixed colonies may be explained by selective contamination of clusters by a fast-sedimenting subset of GM-CFC. Likewise, it was possible that the high Tdr incorporation in the enriched fraction was due to selective contamination of clusters with non-cluster-associated, rapidly growing blasts. We attempted to exclude this possibility by preparing column fractions that contained varying proportions of clusters and single cells (Fig. 4). The purest fractions contained <1% contaminating single cells and these cluster-derived cells incorporated the greatest amount of $[^{3}H]$-Tdr per nucleated cell (Fig. 4). When Tdr incorporation was compared with
the percentage of cells in clusters for each fraction, a direct correlation was found, evidence that clusters were selectively enriched for cycling cells (Fig. 4). The addition of saturating amounts of lung CM (15% vol/vol) doubled TdR incorporation in all fractions (not shown). When a similar experiment was carried out in the presence of lung CM to compare the number of progenitors in different fractions, there was an inverse correlation with the percentage of cells associated with clusters (Fig. 5). This confirmed that clusters were selectively depleted of GM-CFC. In the most purified fraction, 85% of cells were cluster associated and GM-CFC content was depleted about fourfold.

To demonstrate directly that cells attached to RBMM were dividing, adherent clusters were pulsed in vitro for 2–16 h with [3H]TdR. Immunocytochemistry with F4/80 followed by autoradiography showed that 50–100% of nucleated cells overlying RBMM had incorporated [3H]TdR (Fig. 3E). We were unable to detect labeling of RBMM in similar preparations after clustering cells had been washed off. Despite extensive examination, we have been unable to find mitotic RBMM in vitro.

In summary, the clustering cells, but not RBMM, were actively growing, as demonstrated by their high [3H]TdR uptake relative to the nonclustering population. However, since the latter constitutes >90% of the total nucleated population (Table I), the majority of cycling cells were non–cluster-associated (Fig. 4). In contrast, clusters were selectively depleted of GM-CFC. These observations demonstrated that the interaction of RBMM with hematopoietic cells is selective and unlikely to be merely phagocytic.

**Fate and Stability in Culture.** To determine the fate of clusters in vitro, we cultivated 10⁵ cluster-enriched bone marrow cells on glass coverslips for up to 3 d with 10% FBS, without exogenous growth factors. By 24 h, signs of erythropoietic activity were absent whereas myelopoiesis was still occurring, with mature neutrophils present in culture supernatants. At this time clusters began to show signs of dissociation, such that gentle agitation resulted in complete or partial
removal of clustering cells from underlying RBMM. The morphology of RBMM at 24 h in culture was different from that at 2–3 h. Most strikingly, the elaborate branching cellular processes had retracted, plasma membrane spreading was less apparent, and some cells were completely rounded. Another feature at 24 h was the emergence of alkaline phosphatase–bearing fibroblastoid cells from some of the larger clusters. These appeared to have migrated out of clusters to become firmly attached to the coverslips. Immature Mϕ in clusters accompanied the fibroblasts and autoradiographic analysis showed that, in contrast to RBMM, these Mϕ were actively proliferating (not shown). By 48 h, growth of the immature adherent Mϕ was the most prominent feature, myelopoiesis being less evident. At this stage it was difficult to distinguish RBMM from the new Mϕ population by morphological criteria.

In conclusion, RBMM cultivated in clusters showed signs of de-differentiation, including diminished attachment to clustering cells and the loss of their characteristic morphology. This was accompanied by the rapid emergence of a new, immature Mϕ population that was presumably derived from monocytes associated with clusters (see above) as well as from adherent monocytes contaminating the original preparations.

Discussion

In this study we have isolated and characterized the resident Mϕ population within murine femoral bone marrow. Our results show that these cells bear a markedly different phenotype than RPM and that Mϕ in different anatomical sites undergo adaptive differentiation according to their local environment. Although the functions of resident Mϕ in bone marrow are unclear, our overall findings are suggestive of both endocytic and trophic interactions since these cells exist within clusters that are enriched for dividing immature hematopoietic cells.

To isolate resident Mϕ from marrow it was essential to avoid mechanical disruption, which killed these delicate cells. With collagenase, however, the tissue was readily dispersed into a mixture of clusters and single cells. Cells that stained strongly for the Mϕ-specific marker, F4/80, were found exclusively within the clusters and could be extracted from them by selective adherence to glass. In agreement with a previous study on RBMM in situ (22), we found that the RBMM plasma membrane formed extensive projections within clusters, making intimate contact with hematopoietic cells of both myelomonocytic and erythroid lineages. These findings differ from an earlier report (10) which demonstrated that resident Mϕ, stained with acid phosphatase, were associated predominantly with erythroblasts and that immature granulocytes were associated with alkaline phosphatase–positive fibroblastoid cells. One explanation for the differences could be the different methods of Mϕ detection used. Clearly, a lysosomal marker such as acid phosphatase would not reveal elaborate Mϕ plasma membrane extensions that are associated with granulocytes and readily demonstrable with F4/80 (22).

In agreement with the earlier study, however, we found that the alkaline phosphatase–positive stromal cells were largely associated with myeloid clusters, although these clusters often contained erythroid cells as well. Since weakly F4/
80-staining promonocytes and monocytes were enriched in clusters, it is clear
that two distinct Mφ populations exist in close proximity in bone marrow.
Although previous studies (42) have characterized the immature Mφ in cell
susensions of murine bone marrow, little reference has been made to the
mature Mφ population. It is likely that the branching processes of stromal Mφ
within larger clusters are sheared during mechanical dispersion, resulting in cell
death. However, Mφ within erythroblastic islets are probably more robust, since
they are obtained from rodent marrow without enzymatic dispersion (13).

Intimate associations between Mφ and hematopoietic cells have also been
demonstrated in long-term (Dexter-type) bone marrow cultures. In the presence
of anemic mouse serum, erythroblastic islets develop that are similar to those
seen in vivo (5). In its absence, granulocyte production is predominant (3, 4);
immature stages often form intimate clusters with well-spread F4/80+ Mφ (13).
The formation of clusters containing central tissue Mφ is not restricted to hematopoietic systems, but occurs also in
lymphopoietic organs such as the spleen and thymus (43, 44). The best charac-
terized of these is the thymus, from which two distinct types of “rosettes” were
obtained after enzymatic dispersion; one type contained a central Mφ surrounded
by thymocytes while the other contained a central dendritic cell (43).

Our initial step in studying the possible functions of RBMM was to determine
whether their surface phenotype differed from resident tissue Mφ obtained from
the peritoneal cavity. For a variety of markers, we found both quantitative and
qualitative differences between the two populations. For instance, RBMM stained
more intensely for antigens F4/80 and 2.4G2 (IgG1/2b FcR), yet lacked detect-
able Mac-1 (CR3), which is present on monocytes and peritoneal Mφ (25).
Appropriate controls showed that our inability to detect this antigen was not due
to collagenase-mediated proteolysis. Consistent with the immunocytochemistry,
we could not detect complement receptors by rosetting. Our inability to detect
CR3 (and CR1) on RBMM may reflect down-regulation of the receptor molecule
or occupancy of the binding site by endogenous ligand. It is interesting that for
other tissue Mφ examined for Mac-1 expression, the antigen is low or undetect-
able on Kupffer cells (45 and Lee, unpublished results), yet present on microglial
cells (46). In view of the recent findings (47) that Mac-1 and closely related
molecules are important for a variety of functions associated with cellular
adhesion, its absence or presence on certain tissue Mφ populations may be
significant.

The expression of Ia antigens was found to be greater on RBMM than on
RPM. Depending on the mouse strain examined, we demonstrated Ia antigens
on up to 80% of RBMM, and up to 30% showed intense rather than faint
deposition of DAB reaction product. The functional significance of Ia antigens
on these cells is presently unknown, though it is possible that the positive
subpopulation plays a role in mediating certain regulatory functions, as suggested
by in vitro models of hematopoiesis (21). Previous studies have shown that the
expression of Ia antigens on Mφ and mesenchymal cells is largely regulated by
lymphokines, especially γ interferon (IFN-γ) (48), and that with peritoneal Mφ,
high levels of Ia antigen are often associated with an “activated” phenotype (38).
However, the resident nature of RBMM was confirmed by our demonstration
that they did not produce a respiratory burst on challenge with zymosan, a potent stimulator of this function in activated Mφ. Since RBMM showed high phagocytic activity, it is possible that the ability to elicit a respiratory burst is selectively inactivated in situ. Local release of oxygen metabolites could be toxic to attached hematopoietic cells. Further studies are needed to determine whether this and other ligand-induced secretion products (e.g., prostaglandins) are absent from RBMM, and whether such functions are altered in response to lymphokines such as IFN-γ (49).

During the course of these studies we found that 50–90% of RBMM, but not RPM, bound strongly to unopsonized sheep E. This hemagglutinin activity, which, as far as we are aware, has not previously been characterized, appears to be present on certain "fixed" Mφ populations but absent from all "free" populations examined (manuscript in preparation). Thus, in addition to lacking structures present on RPM, RBMM possess at least one and possibly other distinct cell surface components that may be involved in unique functions.

To investigate the possible trophic interactions of RBMM with hematopoietic cells, we initially compared the abilities of clustering and nonclustering cells to incorporate TdR. Our finding that clusters were selectively enriched with cycling cells is analogous to previous observations that thymic Mφ rosettes were enriched with cycling thymocytes (43). We also sought direct evidence that RBMM were associated with actively growing cells. Autoradiography showed that a majority of cells attached to Mφ incorporated [3H]TdR during a 2 h pulse in vitro. In contrast, the RBMM were not labeled, even after a 16 h pulse with [3H]TdR. The low mitotic index of RBMM (<1%) suggests that these cells turn over slowly in situ. Further studies are needed to determine whether RBMM are maintained by self-repopulation or are derived from circulating or locally produced monocytes.

In contrast to their enrichment for cycling cells, clusters were found to contain low numbers of GM-CFC. Since previous studies have shown that myelomonocytic progenitors are actively cycling (50), our results imply that only a subset of proliferating cells become clustered with RBMM in situ. It is possible that GM-CFC cannot bind Mφ or clustering cells or that, if they do, the interaction is labile to collagenase digestion or that an association is prevented for anatomic reasons. In this respect, GM-CFC are thought to be concentrated in subendosteal regions (51), whereas RBMM are distributed uniformly within mouse femoral tissue (10, 22).

Our results clearly demonstrate that RBM interact with hematopoietic cells in a selective and specialized fashion. It is possible that RBMM provide trophic stimuli to the clustering cells in addition to their endocytic activities and other potential regulatory functions. A major aim in future studies will be to characterize Mφ molecules involved in cluster formation and demonstrate the roles they play in hematopoietic regulation.

Summary

In situ studies with the mouse macrophage (Mφ)-specific antibody, F4/80, have shown that resident Mφ in femoral bone marrow (RBMM) form hematopoietic islands with immature myelomonocytic and erythroid cells (Hume, D. A., et al.)
We have isolated these islands (clusters) by collagenase digestion, purified them from single cells by velocity sedimentation, and analyzed their cellular content. The clusters, ranging from 5- to 100 cells, constituted ~7% of the total nucleated cells, and >70% contained at least one strongly staining, F4/80+ central Mφ. In comparison, <26% showed reactivity for alkaline phosphatase, a marker of fibroblastoid reticulum cells. Compared with the nonclustering population, clusters were enriched with RBMM, fibroblastoid cells, and immature hematopoietic cells, but depleted of mature granulocytes and erythrocytes. The RBMM population was purified from other cells in clusters by selective adherence to glass and was compared with resident peritoneal Mφ (RPM) for morphology and the presence of antigens, receptors, and enzymes. RBMM spread more extensively than RPM and frequently extended delicate plasma membrane processes. These and subsequent differences were not attributable to the collagenase treatment. Both Mφ populations stained positively with antibodies F4/80 and 2.4G2 (Fc receptor IgG1/2b), bore mannosyl/fucosyl receptors, and showed reactivity for acid phosphatase and nonspecific esterase I. In contrast to RPM, RBMM had no detectable Mac-1 antigen (CR3) or complement receptors, but bore higher levels of Fc receptors (IgG2a and IgG2b) and Ia antigens. In addition, RBMM possessed a novel hemagglutinin activity for unopsonized sheep erythrocytes, which was not present on RPM. RBMM showed no respiratory burst activity in response to zymosan particles, but ingested them avidly.

The growth properties of clustering and nonclustered populations were compared by measurement of [3H]thymidine incorporation and progenitor assays. Cells in clusters incorporated three- to fourfold more thymidine than nonclustered cells even in the absence of exogenous growth factors, and autoradiography demonstrated that RBMM made contact with proliferating cells. In contrast, the clusters contained over threefold fewer granulocyte/Mφ progenitors compared with nonclustering cells. When clusters were cultivated for up to 3 d, there was rapid outgrowth of monocytes and fibroblastoid cells.

These studies demonstrate that RBMM bear a distinct morphology and phenotype. Their association with cycling immature hematopoietic cells suggests that, in addition to endocytic functions, RBMM may provide trophic stimuli that are important in hematopoietic regulation.

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