Murine Fetal Liver Macrophages Bind Developing Erythroblasts by a Divalent Cation-dependent Hemagglutinin

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Abstract. During mammalian development the fetal liver plays an important role in hematopoiesis. Studies with the macrophage (Mφ)-specific mAb F4/80 have revealed an extensive network of Mφ plasma membranes interspersed between developing erythroid cells in fetal liver. To investigate the interactions between erythroid cells and stromal Mφ, we isolated hematopoietic cell clusters from embryonic day-14 murine fetal liver by collagenase digestion and adherence. Clusters of erythroid cells adhered to glass mainly via Mφ, 94% of which bound 19 ± 11 erythroblasts (Eb) per cell. Bound Eb proliferated vigorously on the surface of fetal liver Mφ, with little evidence of ingestion. The Mφ could be stripped of their associated Eb and the clusters then reconstituted by incubation with Eb in the presence of divalent cations. The interaction required less Ca^{++} than Mg^{++}, 100 vs. 250 μM for half-maximal binding, and was mediated by a trypsin-sensitive hemagglutinin on the Mφ surface. After trypsin treatment fetal liver Mφ recovered the ability to bind Eb and this process could be selectively inhibited by cycloheximide. Inhibition tests showed that the Eb receptor differs from known Mφ plasma membrane receptors and fetal liver Mφ did not bind sheep erythrocytes, a ligand for a distinct Mφ hemagglutinin. We propose that fetal liver Mφ interact with developing erythroid cells by a novel nonphagocytic surface hemagglutinin which is specific for a ligand found on Eb and not on mature red cells.

DURING mammalian embryonic development, hematopoiesis shifts from yolk sac to fetal liver and subsequently to the spleen and bone marrow (19). Macrophages (Mφ)[1] detected by the specific mAb F4/80 (2) are present in murine fetal liver within hematopoietic islets by day 11 of development. These Mφ display long stellate plasma membrane processes which are closely associated with developing erythroid cells (10), suggestive of local cell–cell interactions during erythroid growth and differentiation. Similar Mφ are present in adult bone marrow within clusters of developing erythroid and myeloid cells and after isolation have been found to express unusual characteristics compared with other Mφ populations (6). In particular, these bone marrow stromal Mφ bear a lectinlike hemagglutinin for unopsonized sheep erythrocytes (7), but the possible role of this surface receptor in hematopoietic cell interactions has not been defined.

The early stages of hematopoiesis in the fetus differ from that observed in the adult in that production consists predominantly of erythroid and monocytic cells, with minimal myelopoiesis. Furthermore, distinct generations of red cells are produced in the yolk sac versus fetal liver (17, 19) and the associations of these cells with the local Mφ population also vary (Morris, L., unpublished results). In order to learn more about the possible role of stromal Mφ within the major fetal hematopoietic microenvironment, we have isolated Mφ and associated erythroblasts (Eb) from fetal liver by collagenase digestion and adherence. We report here that murine fetal liver Mφ (FLMφ) avidly bind proliferating Eb by a novel divalent cation-dependent hemagglutinin. This Eb receptor (EbR) is restricted to selected Mφ populations as found in hematopoietic tissues and does not mediate ingestion of bound Eb, suggesting that it could contribute to trophic interactions between stromal Mφ and developing Eb.

Materials and Methods

Animals

Embryos were obtained from the F2 generation of CBA × C57BL/6 matings. Females were inspected daily and the appearance of a vaginal plug designated day 0 of pregnancy. C57BL/6 mice between 8–12 wk of age were used as a source of adult material.

Media and Reagents

RPMI 1640 and Hanks balanced salt solution (HBSS) with or without Ca^{++} and Mg^{++} were purchased from Gibco-Biocult Ltd., Paisley, Scotland. The defined serum-free medium HB102 was obtained from New England Nuclear, Boston, MA. All media were supplemented with 2 mM glutamine, 20 μg/ml gentamycin and 20 mM Hepes buffer (Gibco-Biocult Ltd.). Phosphate buffered saline without Ca^{++} and Mg^{++} (PBS) was ob-

1. *Abbreviations used in this paper*: Eb, erythroblast; EbR, erythroblast receptor; FcR, Fc receptor; FLEb, fetal liver erythroblast; FLMφ, fetal liver macrophage; Fn, fibronectin; FnR, fibronectin receptor; FRC, fetal circulating red cells; Mφ, macrophage; ME, mouse erythrocyte; SE, sheep erythrocyte; SER, sheep erythrocyte receptor.

tained from Oxoid Ltd., Basingstoke, Hampshire, United Kingdom. FBS was purchased from Sera-Lab Ltd., Crawley Down, Sussex, UK and heat inactivated at 56°C for 30 min. Collagenase, type 1, was bought from Boehringer Corp., Lewes, East Sussex, UK, and DNase, type 1, from Sigma Chemical Company, Ltd., Poole, Dorset, UK.

Other enzymes were obtained and used at the concentrations shown unless noted otherwise. From Sigma Chemical Company, Ltd.: trypsin, type IX, 100 μg/ml; *Bacillus subtilis* protease, type VIII; and *Staphylococcus griseus*, type XIV (Pronase E), 800 μg/ml; elastase and α-chymotrypsin, 200 μg/ml. From Boehringer Corp.: pronase and neutral protease (dispase), 800 μg/ml; phospholipase D, 200 μg/ml. Phosphatidyl-inositol-specific phospholipase C, 20 μg/ml was a gift of Dr. M. Low, Oklahoma Medical Research Foundation, Oklahoma City, OK; neuraminidase from *Vibrio cholerae*, Calbiochem-Behring Corp., La Jolla, CA, was used at 0.01 Behringwerke U/ml, unless noted; chondroitinase ABC and hyaluronidase, gifts of Prof. H. Muir, Kennedy Institute, University of London, UK, were used at 0.02 and 0.4 U/ml, respectively.

Substances screened for inhibition of cluster formation were obtained and used as follows: D-galactose (100 mM), lactose (10 mM), *N*-acetyl D-galactosamine (100 mM), asialofetuin (1 mg/ml), mannan (5 mg/ml) all from Sigma Chemical Company, Ltd.; *N*-acetylneuraminyllactose from bovine colostrum (20 mM) and neuraminic acid (50 mM) from Boehringer Corp. Mannosylated BSA, galactosylated BSA, fucosylated BSA, and acetylated glucose BSA (with 33–37 mol of sugar per mole protein, 200 μg/ml) were gifts from Dr. P. Stahl, Washington University, St. Louis, MO. The ganglioside GDIa (50 μg/ml, 29 μM) was provided by Dr. J. Mellanby, Oxford University, UK.

Other reagents included heparin 10,000 U/ml, cycloheximide 25 ng/ml, EDTA, 10 mM, EGTA, 6 mM, all from Sigma Chemical Company Ltd.; hyaluronic acid 1 mg/ml (a gift of Prof. H. Muir) the fibronectin (Fn) peptides GRGDSPC and GRGDSP 100 μg/ml, a gift of Dr. E. Piersbacher (Salk Institute, La Jolla, CA); [6-^{3}H]thymidine (2 Ci/mMol), from Amersham International PLC, Amersham, Buckinghamshire, UK; $CaCl_2$, $MgCl_2$, and $MgSO_4$ from BDH Chemicals Ltd., Poole, Dorset, UK.

Antibodies

Rat anti-mouse mAb with specificities shown were used as supernatants at saturating concentrations: F4/80 (Mφ); M1/70, CR3 on neutrophils, Mφ and natural killer cells (27); 2.4G2, trypsin-resistant FcR (29) and ECCD-2 (E-Cadherin, Dr. M. Takeichi, Kyoto University, Kyoto, Japan). Polyclonal antisera against Fn and Fn receptors (FnR) were obtained as follows: rabbit polyclonal antisera against rat plasma Fn (Dr. R. C. Hughes, National Institute of Medical Research, London and Dr. R. O. Hynes, Massachusetts Institute of Technology, Boston, MA); goat anti-hamster FnR ab (Dr. R. L. Juliano, University of Texas Medical School, Houston, Texas), and rabbit anti-baby hamster kidney FnR (Dr. G. Tarone, University of Torino, Italy).

Preparation of Fetal Liver Cultures and Macrophages

Pregnant females (embryonic day [d]13–d15, usually d14) were killed by cervical dislocation and fetuses were removed aseptically into cold PBS. Fetuses were washed three times in PBS and the dissected livers placed in prewarmed 0.05% collagenase and 0.002% DNase in RPMI, (usually 10 livers per 40 ml enzyme), and digested for 1 h at 37°C on a rotating wheel at 30 revolutions per minute. Tissue dissociation was completed by gentle pipetting through a wide bore plastic pipette. FBS was added, final concentration 10%, and the suspension left for 5 min to allow large fragments to settle. Cells were washed three times in RPMI at 300 *g* for 10 min and resuspended in RPMI plus 10% FBS at 2×10^7 nucleated cells/ml. 100 μl aliquots were placed on precleaned glass coverlips for 20 min at 37°C in 5% CO_2 and flooded with RPMI plus 10% FBS. Cells were then incubated further for 4–6 h. Adherent cells with attached erythroid clusters were obtained by dipping coverslips three times each in three beakers of RPMI to remove nonadherent cells. To reveal underlying Mφ, coverslips were rinsed in PBS, incubated in PBS for 30 min at room temperature, and "stripped" by repeated and direct gentle flushing, using PBS and a wide bore plastic pipette. Large epithelioid cell aggregates often detached during this procedure leaving a population of adherent Mφ which respread extensively when placed in RPMI.

Erythroid Ligands

Fetal Liver Erythroblasts (FLEb). Collagenase-digested or mechanically disrupted fetal liver was used interchangeably as a source of ligand with no apparent difference. Cells were washed three times at 300 *g* for 10 min in RPMI and 2×10^7 plated per 100-mm tissue culture dish in 10% FBS. After 4 h at 37°C, dishes were flushed gently to recover nonadherent and most clustered red cells. These were washed in PBS before reincubation with stripped Mφ as described below. The total nonadherent population was analyzed after cytocentrifugation.

Fetal Circulating Red Cells (FRC). 14-d-old embryos were dissected with placenta and yolk sac intact. After extensive washing in PBS, the yolk sac and placenta were removed and the embryos transferred to a dish containing PBS plus 10 U/ml heparin and allowed to bleed. The heads were severed to facilitate bleeding. After 15 min, cells were collected and washed in PBS before use.

Adult Erythrocytes. After CO_2 asphyxiation, mouse erythrocytes (ME) were collected by cardiac puncture in a heparinized syringe. Sheep erythrocytes (SE) were purchased from Gibco-Biocult Ltd.

Rosetting Assays

Assays were done in media with (RPMI, HBSS) or without (PBS, HBSS) divalent cations. Adherent cells were rinsed three times in the assay medium before transfer to a 24-well tray. Erythroid ligands were washed four times in PBS at 300 *g* for 10 min and resuspended in assay medium. 50 μl of diluted cells (2×10^7/ml of FLEb or FRC, 5% vol/vol SE and ME) were added to various adherent Mφ preparations and incubated for 30 min at 37°C. Unbound ligand was removed by dipping coverslips four times each in four beakers of RPMI and the cells fixed in 0.25% vol/vol glutaraldehyde. Reagents listed above were tested for inhibition of rosetting and the concentration listed was also the highest that gave no inhibitory effect. All reagents were dissolved in assay medium, preincubated with stripped Mφ for 30 min, and incubated in the continued presence of inhibitors except where noted.

Enzyme Treatments

Unstripped cultures as well as stripped Mφ and ligand preparations were treated with various enzymes. The concentrations shown were the highest at which no effects were noted, including cytotoxicity. Enzymes were dissolved in RPMI, except where stated, and incubated with cells for 60 min at 37°C. All cells were then incubated in 20% FBS in RPMI for 30 min and washed well in the same medium before assay.

Immunocytochemistry

Cells on coverslips or as cytocentrifuge preparations were fixed in 0.25% glutaraldehyde for 10 min, rinsed in PBS, and incubated in 10% FBS in PBS for 30 min. Antigens were detected by immunoperoxidase labelling using an avidin-biotin system (15).

Scoring

Cells were analyzed after labeling with specific antibody or after Giemsa or hematoxylin staining. Mφ were identified morphologically by phase contrast microscopy or by F4/80 labeling. Rosetted cells were scored as binding of 2 or more FLEb or FRC, and 5 or more of the smaller ME or SE. Assays were done in duplicate and at least 200 Mφ scored per coverslip. FLEb and FRC were analyzed after Giemsa or hematoxylin staining to determine their stage of differentiation.

Scanning Electron Microscopy

Clusters were fixed in 2.5% glutaraldehyde (EM grade) in a 0.1 M sodium cacodylate, 1% sucrose buffer for 30 min at room temperature. Samples were dehydrated, critical-point dried, and coated with 150 Å gold. A JEOL 100CX scanning electron microscope was used for viewing.

Results

Isolation of Hematopoietic Clusters and Fetal Liver Macrophages

Hematopoietic clusters were isolated from pooled d14 fetal livers by collagenase digestion and mechanical disruption. The total digest consisted mostly of free, nucleated erythroid cells and a smaller population of clustered erythroid cells and F4/80$^+$ Mφ (Table I). Enrichment of Mφ and clustered

Table I. Isolation and Characterization of Mφ–Erythroid Cell Clusters from Embryonic Day-14 Murine Fetal Liver by Collagenase Digestion and Adherence

Cell type	Cellular composition of total fetal liver digest*	Nature of adherent cells associated with Eb		
	Percent of all nucleated cells	Percent of total‡	Percent of each cell type that bound Eb§	Eb bound per cell‖
Erythroid cells	92			
Mφ ($F4/8^+$)–	2.6			
total in clusters	(2.1)	50	93	19 ± 11
Epithlioid aggregates¶	1	28	ND	ND
Myeloid clees	1			
Megakaryoblasts	0.4			
Fibroblasts		18	15	0.7 ± 1.7
Other	2.8	4	9	0.4 ± 1.1

* 1–2 × 10^7 nucleated cells were recovered from each fetal liver. 1,000 cells counted from each of two cytocentrifuge preparations. Fibroblasts cannot be distinguished from "others" in these preparations. Nuclei and anucleate E were excluded in this table. The erythroblast population included a proportion of other hematopoietic blastlike cells.

‡ 1,000 cells counted on each of two coverslips. The composition of adherent cultures varied somewhat between experiments, results show one experiment representative of three.

§ 100–200 of each cell type counted on duplicate coverslips.

‖ Results show average ± SD.

¶ Shows percent aggregates of prehepatocytes that could not be scored as single cells and often contained trapped Mφ and Eb.

erythroid cells was achieved by adherence to glass for 4–6 h. When preparations were viewed by phase contrast microscopy, this procedure yielded numerous aggregates of refractile hematopoietic cells (Fig. 1 *a*), which obscured a population of underlying Mφ, the latter comprising ~50% of the adherent cell population (Table I). The plasma membrane of these extensively spread Mφ could be revealed by immunocytochemistry using the mAb F4/80 (Fig. 1 *b*). In addition to mature Mφ and isolated monocytes, which were also $F4/80^+$, there were variable aggregates of epithelioid prehepatocytes, isolated spindly fibroblasts, and ill-defined mesenchymal cells, which were all $F4/80^-$. The epithelioid cells were often associated with refractile lipid. In >30 experiments, 90–98% of stromal Mφ were present in hematopoietic clusters, which contained 19 bound cells per Mφ (Table I). Apart from low levels of binding (<1 per cell) by a proportion of fibroblasts (<<22% in three different experiments), isolated $F4/80^-$ adherent cells did not bind hematopoietic cells. The Mφ-associated cells, which did not adhere directly to glass under these conditions, were almost exclusively of the erythroid series. Myeloid cells were rare compared with adult bone marrow clusters prepared by similar methods (6). Erythroid cells were at different stages of development, but often appeared synchronous in individual clusters.

The majority of bound erythroid cells (~85%) were Eb, though more mature anucleate stages (~9%) and pyknotic erythrocyte nuclei (~6%) were also present. Light and scanning electron microscopy (Fig. 1 *h*) revealed erythroid cells nestling in cuplike, loosely applied folds of Mφ plasma membrane, with prominent $F4/80^+$ ruffles. A striking feature was the absence of ingestion of bound Eb by FLMφ. Eb mitoses were evident and Eb DNA synthesis was confirmed by autoradiography after labeling with [^{3}H]thymidine (Fig. 1 *g*).

Control experiments established that mechanical dispersion without collagenase digestion yielded fewer clusters and stromal-type Mφ. Collagenase was superior to other proteolytic enzymes, including dispase, pronase, and trypsin and addition of hyaluronidase offered no advantage. Cluster formation was not an artifact of digestion or adherence since they were observed at all stages of isolation, with or without enzyme.

Stripping of FLMφ and Reconstitution of Hematopoietic Clusters

To reveal underlying stromal Mφ, coverslips were incubated in Ca^{++}- and Mg^{++}-free PBS for 30 min at room temperature and washed gently by direct flushing. Almost all Eb were detached by this procedure whilst pyknotic nuclei were more difficult to remove. Cytocentrifuge preparations of detached cells revealed basophilic, polychromatic, and orthochromatic Eb and a few anucleate erythrocytes. The adherent Mφ resembled pancakes as a result of extensive symmetrical spreading and contained large perinuclear vacuoles as well as numerous small vesicles (Fig. 1 *c*). The extent of Mφ spreading depended on the presence of divalent cations. Mφ remained adherent in PBS, with spiky, retracted plasma membrane processes.

Erythroid–Mφ clusters were reconstituted by adding nonadherent cells to Mφ monolayers in the presence of divalent cations. For this purpose erythroid cells, which were present in large excess, were depleted of adherent cells and clusters by incubation for 4–6 h in tissue culture dishes. After incubating Mφ with ligand in RPMI, phase-contrast (Fig. 1 *d*) and cytochemical examination (Fig. 1 *e*) revealed striking reformation of erythroid–Mφ clusters. In >30 experiments 61–89% of Mφ bound 2–30 Eb whereas only trace binding occurred to occasional $F4/80^-$ cells. Binding was similar to that observed in unstripped clusters, involved mainly Eb (~84%), a few anucleate erythrocytes (3%), and pyknotic nuclei (~13%), and there was little ingestion. After reconstitution, the Eb population was more heterogeneous than unstripped individual clusters.

Requirements for Cluster Formation

Binding of Eb to FLMφ was independent of temperature (4°C, room temperature, 37°C), but depended on divalent cations (Fig. 1, *e* vs. *f*), either Ca^{++} or Mg^{++} (Fig. 2, Table

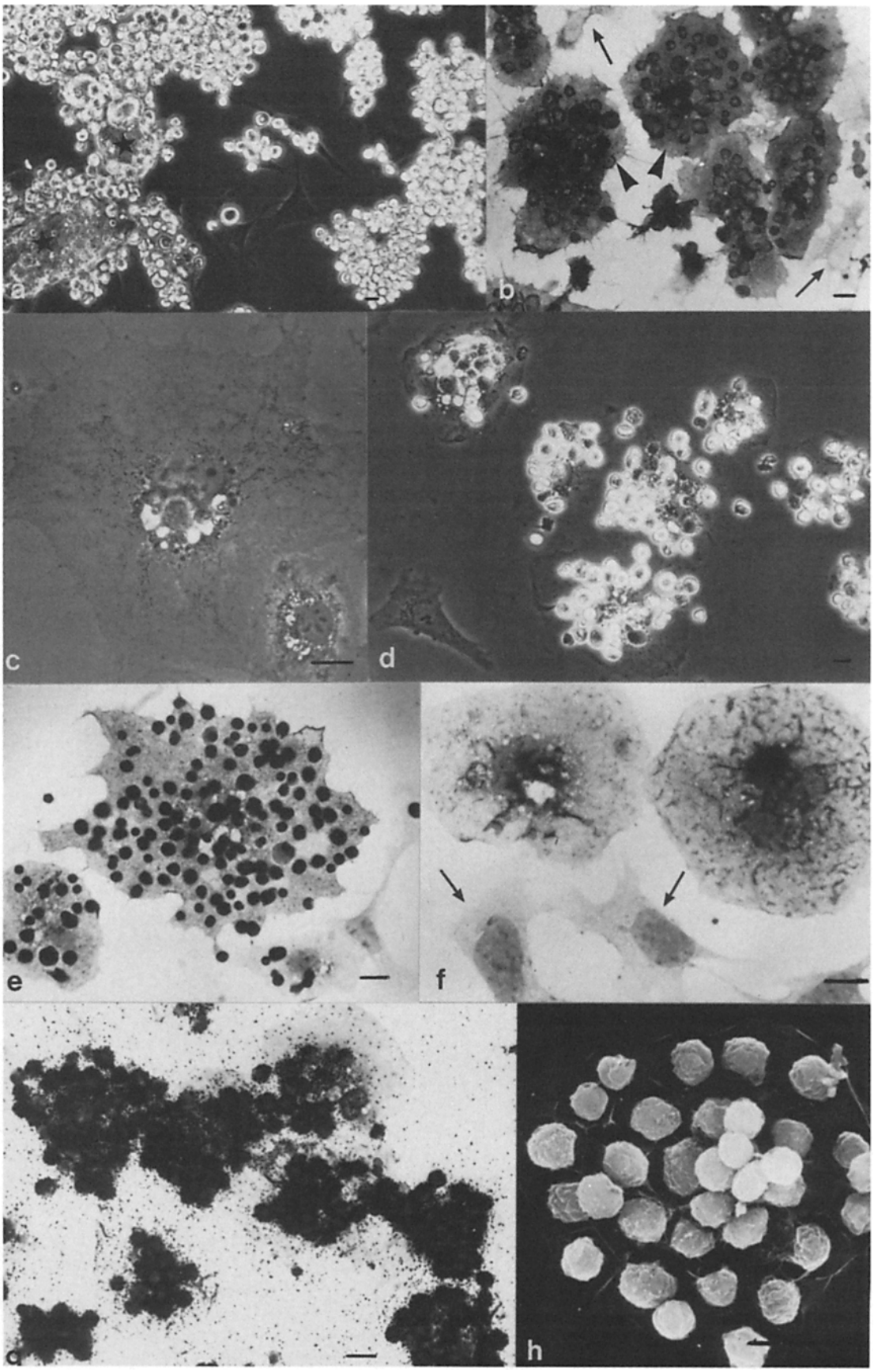

Figure 1. Clusters and Mφ from fetal liver after collagenase digestion and enrichment by adherence to glass. (*a*) Phase-contrast micrograph after 4-h culture. Numerous clusters of refractile hematopoietic cells and occasional large aggregates of lipid-containing prehepatocytes (★) are evident. Unclustered well-spread fibroblasts are also present. (*b*) Immunocytochemical analysis after 4-h adherence. Large well-

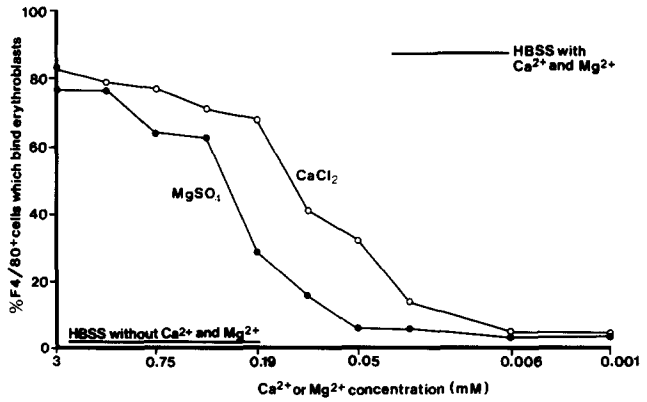

Figure 2. Effect of Ca^{++} or Mg^{++} concentration on Eb binding to FLMφ. Stripped Mφ in $CaCl_2$ (○) or $MgSO_4$ (●) were rosetted with ligand for 30 min at 37°C. All dilutions were in Ca^{++}- and Mg^{++}-free HBSS, including Eb suspensions. The positive control was Ca^{++}- and Mg^{++}-containing HBSS (87%). Mφ that bound two or more Eb were scored after F4/80 staining. Free nuclei bound in absence of divalent cations and have been omitted from analysis. 200 Mφ were counted on each coverslip. Similar results were obtained in three independent experiments.

II). Ca^{++} was more efficient than Mg^{++} (100 vs. 250 μM, respectively, to give 50% of maximal binding). The chelating agents EDTA or EGTA prevented binding in the presence of Ca^{++} and Mg^{++} and their action could be overcome by excess divalent cations. Pretreatment of either FLMφ or Eb with chelator did not affect subsequent binding in RPMI, indicating that cations were required during cell interaction and that the effects were fully reversible (not shown). It was also noted that a few pyknotic nuclei with a small rim of cytoplasm bound to FLMφ in the absence of divalent cations.

Trypsin treatment of FLMφ abolished their ability to bind Eb (Fig. 3 *a*), whereas pretreatment of ligand had no effect (not shown). The effects of trypsin were dose related and not influenced by the presence or absence of Ca^{++}. Mφ were not detached from glass by trypsin and recovered the ability to bind Eb when cultivated for 1–2 d in serum-free (Fig. 3 *b*) or serum-containing medium (not shown). After recovery, binding of Eb was still dependent on divalent cations. To determine whether recovery of Eb binding involved protein synthesis we evaluated the effect of cycloheximide. Fig. 3 *b* shows that FLMφ treated with low concentrations of cycloheximide did not recover the ability to bind Eb. Control assays showed that cycloheximide treatment had no effect on binding of Eb by untrypsinized Mφ and did not inhibit FcR-mediated ingestion of EIgG by FLMφ (not shown).

Characterization of Erythroid Ligand

The above experiments indicated that the Eb ligand was not sensitive to trypsin. In further experiments (not shown) the erythroid ligand was also resistant to pronase, dispase, pancreatic elastase, α-chymotrypsin, phospholipase C and D, hyaluronidase, and chondroitinase ABC. Since the binding of sheep erythrocytes by adult bone marrow Mφ is abolished by neuraminidase treatment of the ligand (7), we examined the effect of neuraminidase on Eb binding to FLMφ. In contrast with SE receptor (SER) activity, neuraminidase treatment did not inhibit binding of Eb.

To investigate further the nature of the erythroid cells which were able to bind to FLMφ, fetal, and adult mouse blood cells were compared with fetal liver nonadherent cells as ligand (Table II). Erythrocytes and yolk sac–derived nucleated red cells that persist in fetal blood, as well as adult erythrocytes all bound far less well (<13% of Mφ rosetted) than FLEb, with or without divalent cations. Occasional free nuclei, presumably derived from fetal orthochromatic Eb, bound to Mφ after incubation. Taken with earlier experiments these results indicated that immature FLEb were the predominant ligand-bearing cell.

Relationship of EbR to Other Mφ Receptors

The above experiments indicated that FLMφ express a hemagglutinin for fetal Eb. Since freshly isolated adult bone

Table II. Binding of Various Murine Erythroid Cells to Adherent Fetal Liver Mφ

	Fetal liver Eb		Fetal blood		Adult blood	
Treatment	Mφ rosetted	Cells bound/ 100 Mφ	Mφ rosetted	Cells bound/ 100 Mφ	Mφ rosetted	Cells bound/ 100 Mφ
	%		%		%	
Intact clusters	97	2,016	–	–	–	–
Reconstituted clusters						
+ Ca^{++}/Mg^{++}	81	1,866	11	106	13	157
– Ca^{++}/Mg^{++}	3	189	6	26	12	100

400 Mφ counted were in duplicate preparations. The fetal liver ligand contained 87% Eb, 13% anucleate cells, and 0.3% nuclei; the fetal blood 23% Eb and 77% anucleate cells; and the adult blood 100% mature erythrocytes. Similar results were obtained in two independent experiments.

spread (*arrowheads*) F4/80$^+$ Mφ are present beneath attached Eb, which appear dark as a result of crystal violet counterstain. Unclustered fibroblasts (*arrows*) were F4/80$^-$ (cf. *f*). (*c*) The underlying Mφ are revealed after removal of clustering hematopoietic cells. By phase-contrast microscopy these are large and well spread with phagocytic inclusions. (*d*) Reconstitution of stripped Mφ with erythroid cells in presence (*d* and *e*) or absence (*f*) of divalent cations. Clusters formed exclusively with Mφ and only in the presence of divalent cations. Note heterogeneous mixture of red cells on individual Mφ. (*g*) Autoradiograph showing intense and synchronous incorporation of [^{3}H]-thymidine into clustering Eb. 4-h adherent cultures were pulsed for 2 h and stained with F4/80. (*h*) Scanning electron micrograph shows a single Mφ with Eb nestling within folds of plasma membrane. Bar, 10 μm.

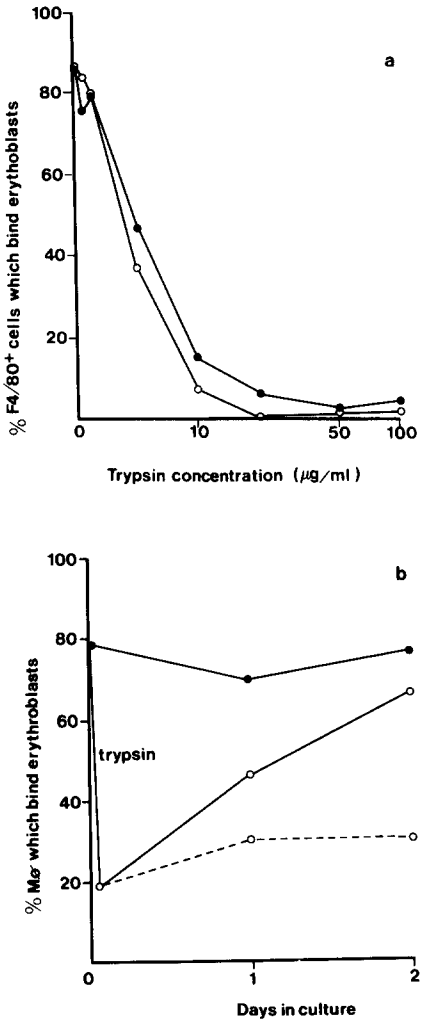

Figure 3. Effect of trypsin treatment of FLMφ on rosette formation. (*a*) 4-h adherent cultures treated in the presence of $CaCl_2$ (○) or EDTA (●) for 60 min at 37°C. After inactivation and removal of enzyme, rosetting with Eb was carried out in the presence of divalent cations. 200 Mφ were counted on each coverslip. Similar results were obtained in three independent experiments. (*b*) Re-expression of EbR after treatment of Mφ with 50 μg/ml trypsin. Cells were cultivated in HB102 medium in the absence of serum and assayed with fresh ligand 1 and 2 d after treatment. Untrypsinized cells maintained receptor levels (●). After trypsin treatment and loss of EbR (-○-), Mφ recovered high levels of activity after 2-d cultivation. Recovery was inhibited by 25 ng/ml cycloheximide (--○--). No difference in control levels of binding was seen in untrypsinized cells cultivated in cycloheximide (not shown). Cells remained viable as judged by FcR-mediated binding and phagocytosis of EIgG (not shown). 200 cells were counted on duplicate coverslips. The results show one experiment representative of two.

marrow stromal Mφ are able to bind unopsonised sheep erythrocytes via the SER (6, 7) we examined binding of this ligand by FLMφ. Only ~3% of freshly isolated FLMφ bound SE, in the presence or absence of divalent cations, indicating that Eb binding was mediated by a distinct hemagglutinin.

Other known Mφ receptors were shown not to mediate binding of mouse fetal Eb to FLMφ by use of appropriate mAb or glycoconjugate inhibitors (see Materials and Methods for reagents tested and Table III). These included FcR (2.4G2), CR3 (M1/70), and the lectin-like mannosyl-fucosyl-receptor (28; mannose-BSA, fucose-BSA) and galactosyl-R (25; galactose-BSA, *N*-acetyl D-galactosamine). Various mono- and disaccharides, glycosaminoglycans, and a range of phospho- and glycolipids (not shown) failed to yield a specific inhibitor for the FLMφ hemagglutinin.

FnR have been implicated in Ca^{++}-dependent binding of mouse Eb and of erythroleukemia cells to Fn-coated dishes (9, 21) and human monocytes/Mφ are known to express receptors for Fn (14, 23). However polyclonal antibody directed against rat plasma Fn (18) or against hamster and rodent FnR (5, 9) did not inhibit Eb binding to FLMφ, nor did peptides that are known to block the Fn cell-binding site (22). Another divalent cation-dependent embryonic cell adhesion molecule (E-cadherin) has been described on murine liver epithelial cells (26). A rat mAb (ECCD-2) that blocks this receptor had no effect on the binding of Eb to FLMφ. Taken together these experiments indicated that the ability of FLMφ to bind Eb was mediated by a distinct divalent cation-dependent haemagglutinin.

Discussion

The fetal liver plays a key role during midgestation production of erythroid cells and may contribute to the turnover of earlier yolk sac-derived forms. Previous immunocytochemical analysis indicated the concomitant appearance of mature Mφ intimately associated with islets of erythroid cells in fetal liver. In this study we have isolated intact hematopoietic clusters and their associated Mφ from fetal liver, characterized the nature of interactions between developing Eb and these Mφ, and provided evidence for the existence of a specific divalent cation-dependent Mφ receptor responsible for binding of Eb. Since Eb are not ingested but proliferate vigorously on the surface of fetal liver Mφ, it seems likely that binding to Mφ could influence erythroid cell development.

Isolation and Characterization of Hematopoietic Clusters and FLMφ

Although only a small proportion of total erythroid cells were clustered with FLMφ after collagenase digestion, the ability of these Mφ to adhere and spread on a variety of substrata provided an efficient method to isolate the Mφ and their associated Eb. Distinctive plasma membrane antigen markers such as F4/80 confirmed morphologic identification of Mφ which were much larger than the immature monocytes. Other adherent cells included aggregates of epitheloid prehepatocytes and isolated fibroblasts, which showed only low levels of interaction with Eb. The predominant hematopoietic cells found on the surface of Mφ were Eb that appeared to nestle in cuplike folds of ruffled, $F4/80^+$ plasma membrane processes. These Eb were heavily and often synchronously labeled with [^{3}H]thymidine, and mitoses confirmed that they proliferated on the Mφ surface. When Eb were detached from Mφ by washing in divalent cation-free media, the FLMφ remained adherent suggesting that their adhesion, but not spreading, is mediated by a divalent cation-independent mechanism. Fetal liver Mφ in the presence of divalent cations spread to a remarkable extent compared with fetal liver monocytes and adult Mφ populations

Table III. Inhibitors of Known Receptors that Did Not Affect Binding of Erythroblasts to Fetal Liver Macrophages

Receptor	Inhibitor	Highest concentration tested	Reference
SER	GDla	50 μg/ml	6, 7
	Neuraminidase	0.01 U/ml	
	N-acetylneuraminyl-lactose	20 mM	
FcR(IgG1/2b)	2.4G2 mAb	Saturating concentrations	29
CR3	M1/70 mAb	Saturating concentrations	27
MFR	Mannosylated BSA	200 μg/ml	28
Galactosyl-R	*N*-acetyl D-galactosamine	100 mM	25
FnR	Fn antisera	Saturating concentrations	18, 5, 9, 22
	FnR antisera	Saturating concentrations	
	Fn peptides GRGDSP	100 μg/ml	
	GRGDSPC	100 μg/ml	
E-cadherin	ECCD-2 mAb	Saturating concentrations	26

and often contained prominent phagocytic vacuoles indicative of active endocytic activity. Other characteristic Mφ markers were also present, e.g., Fc receptors and the ability to ingest opsonized sheep erythrocytes (not shown).

Nature of FLMφ-Eb Interactions

The requirement for divalent cations for binding made it possible to dissociate and reconstitute clusters in vitro. Both Ca^{++} and Mg^{++} sufficed for binding, although Ca^{++} was two- to threefold more potent on a molar basis. Divalent cations were required during binding and did not influence the susceptibility of the Mφ receptor to proteolysis, unlike other cell adhesion systems (8, 13, 16). The susceptibility of the Mφ binding activity to low concentration of trypsin, its re-expression in culture, and sensitivity to cycloheximide provided evidence that the Mφ hemagglutinin is a protein synthesized by FLMφ and is not adsorbed. By contrast, the Eb ligand was resistant to all forms of proteolytic and other treatments used.

Several lines of evidence indicated that Eb binding to FLMφ is mediated by a hemagglutinin which is different from that described on adult bone marrow Mφ (7). Unlike binding of Eb, the SER is divalent cation independent and highly sensitive to neuraminidase treatment of the SE, which had no effect on binding of Eb to FLMφ. Moreover, FLMφ did not bind SE. Our unpublished observations also indicate that binding of Eb by FLMφ is not sensitive to potent inhibitors of SER activity including a specific mAb and specific gangliosides (7). These studies indicate that stromal Mφ in hematopoietic tissues are able to express two distinct hemagglutinins that are independently regulated on fetal liver and adult bone marrow Mφ.

The erythroid ligand has not been defined, although it is apparently present on mouse fetal Eb and not on circulating mouse FRC or adult ME. Eb from adult mouse spleen and nucleated erythroid and myeloid cells from adult bone marrow also bind to FLMφ in a divalent cation-dependent manner (Crocker, P. R., unpublished data). Furthermore, Mφ isolated by collagenase digestion from adult murine bone marrow are able to bind fetal Eb by a divalent cation-dependent hemagglutinin, unlike Mφ obtained from the peritoneal cavity (Morris, L., unpublished data). These studies indicate that EbR-like hemagglutinins may be involved in hematopoietic interactions of stromal Mφ in both the fetus and adult. Since binding is a feature of developing Eb rather than of more mature stages and since binding does not mediate ingestion, it is unlikely on present evidence that the EbR plays a role in clearance of senescent cells.

A wide range of potential inhibitors failed to block binding of Eb to FLMφ, ruling out involvement of several known Mφ receptors. Since a specific inhibitory anti-mouse FnR mAb was not available, our negative studies with Fn peptide and cross-reacting antibody to other species' Fn and FnR cannot be regarded as definitive. However, the Mφ specificity of Eb binding and protease resistance of the ligand on Eb provide further evidence against a role for Fn in our system.

Role of Mφ–Eb Interactions

Erythroblastic islands with central Mφ have been described previously, notably by Bessis and his colleagues (3, 4) and in erythropoietic Dexter cultures, developing red cells associate with stromal Mφ (1). There has however, been little work to characterize the surface molecules involved or to investigate the possible trophic interactions between Mφ and developing Eb. These could include local production of erythropoietin (12, 24), provision of iron and other nutritional requirements, and production of monokines to promote or inhibit erythroid growth and differentiation (20). There is some evidence that erythroid differentiation in yolk sac is incomplete, compared with that observed in fetal liver (17) and since Mφ are not seen in similar clusters in yolk sac in situ (Morris, L., unpublished data), it is plausible that Mφ

in each site contribute to the observed differences in terminal differentiation. Another important contribution of Mφ to mammalian erythropoiesis is the removal of free nuclei. In the present studies we have observed free nuclei with a rim of cytoplasm on the surface of FLMφ and these resisted detachment by chelating agents, suggesting that they bind via a distinct mechanism. Some of the phagocytic inclusions could be derived from such nuclei. There are precedents for Mφ to discriminate between various bound particles, ingesting some while retaining others at the surface (11). Although our present observations are consistent with highly selective local trophic as well as clearance functions for stromal Mφ, it remains to be shown that an association with Mφ is essential for erythroid growth and differentiation in vivo.

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