Extruded Erythroblast Nuclei Are Bound and Phagocytosed by a Novel Macrophage Receptor

By Li-Bo Qiu, Harriet Dickson, M.A. Nasser Hajibagheri, and Paul R. Crocker

Resident bone marrow macrophages (RBMM) play an important role in clearance of nuclei extruded from late-stage erythroblasts (Eb). To investigate the nature of macrophage receptors involved in this process, extruded erythroblast nuclei (EEN) were purified by cultivation of erythroblasts with erythropoietin, followed by density gradient centrifugation. By electron microscopy, the majority of free nuclei had an intact plasma membrane. EEN bound avidly to RBMM in a divalent cation-independent manner at both 4°C and 37°C. The use of specific monoclonal antibodies (MoAbs) and inhibitors showed that this adhesive interaction was not mediated by previously characterized macrophage receptors involved in recognition of either developing hematopoietic cells or apoptotic cells. The EEN receptor was expressed on resident macrophages isolated from murine bone marrow, spleen, lymph node, and peritoneal cavity, but was completely absent from alveolar macrophages. Despite high levels of EEN binding to RBMM, few were phagocytosed even after prolonged culture. Phorbol myristate acetate (PMA) was found to stimulate phagocytosis, suggesting that this is a regulated process. These findings indicate that EEN are recognized by a novel macrophage receptor and that recognition may be triggered during the membrane remodeling that accompanies enucleation.

In adult mammals, the last step in development of the erythroid lineage is the production of erythrocytes by extrusion of nuclei from late-stage erythroblasts (Eb). In this process, the nucleus moves to one pole of the Eb and is then actively expelled, together with an intact plasma membrane and a small amount of cytoplasm. The precise mechanisms that control this expulsion are unknown. However, it is an intrinsic property of the Eb, as enucleation occurs in isolated Eb cultured in the presence of erythropoietin and actin polymerization is required as shown by the inhibition of nuclear extrusion in the presence of cytochalasin D. In addition, immunofluorescence studies have shown that enucleation is accompanied by cytoskeletal reorganization involving actin, spectrin, and microtubules, all of which become selectively localized within the nascent reticulocyte.

In the bone marrow, developing Eb, from the proerythroblast stage onwards, are in close contact with central resident macrophages. These associations can be observed in sections of bone marrow as distinct anatomical structures that have been described as erythroblastic islands. After enucleation, the nucleus is rapidly phagocytosed by macrophages, whereas the reticulocyte membrane and a small amount of cytoplasm are bound by RBMM in a divalent cation-independent manner. EEN bound avidly to RBMM in a divalent cation-independent manner at both 4°C and 37°C. The use of specific monoclonal antibodies (MoAbs) and inhibitors showed that this adhesive interaction was not mediated by previously characterized macrophage receptors involved in recognition of either developing hematopoietic cells or apoptotic cells. The EEN receptor was expressed on resident macrophages isolated from murine bone marrow, spleen, lymph node, and peritoneal cavity, but was completely absent from alveolar macrophages. Despite high levels of EEN binding to RBMM, few were phagocytosed even after prolonged culture. Phorbol myristate acetate (PMA) was found to stimulate phagocytosis, suggesting that this is a regulated process. These findings indicate that EEN are recognized by a novel macrophage receptor and that recognition may be triggered during the membrane remodeling that accompanies enucleation.

One possibility is that differences in membrane composition form a molecular basis for the macrophage recognition events described above. Previous studies have shown that the plasma membrane surrounding the nucleus is selectively enriched in glycoconjugates recognized by concanavalin A (Con A). The nuclear plasma membrane also binds weakly to positively charged colloidal iron oxide, in contrast to the reticulocyte membrane. This reduced negative charge of extruded erythroblast nuclei (EEN) is a possible explanation for why they are avidly bound and phagocytosed by macrophages in vivo.

In previous studies, macrophages isolated from hematopoietic tissues were found to express two distinct receptors for developing hematopoietic cells, the erythroblast receptor (EBR) and sialoadhesin. EBR mediates divalent cation-dependent binding of Eb in vitro, whereas sialoadhesin is a divalent cation-independent receptor that is believed to interact selectively with myeloid precursors.

So far, no studies have investigated the nature of receptors for EEN on resident bone marrow macrophages (RBMM). A major technical obstacle is the isolation of sufficient numbers of purified EEN to permit direct binding assays. In the present study, we first developed a method in which a relatively large and homogeneous population of fluorescently labeled EEN could be isolated from Eb cultured in the presence of erythropoietin. Binding assays were then performed with RBMM. Our studies showed that EEN were avidly bound by RBMM in a divalent cation-independent manner. The failure of a range of specific antibodies and inhibitors to block this interaction, as well as the characteristics of binding and the pattern of expression on various macrophage populations, suggest that the EEN receptor (EENR) is a novel macrophage adhesion and phagocytosis receptor.

MATERIALS AND METHODS

Animals. Male or female Balb/c mice were bred at Clare Hall, Imperial Cancer Research Fund (ICRF; South Mimms, Hertfordshire, UK) and used at 8 to 12 weeks of age.
Media and reagents. Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640, and phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺ were obtained from Clare Hall. Fetal calf serum (FCS) was purchased from Advanced Protein Products Ltd (Brookmoor, UK) and was routinely heat-inactivated for 30 minutes at 56°C. Recombinant mouse erythropoietin, collagenase, and DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) were obtained from Boehringer Mannheim (Lewes, UK). Vibrio cholerae sialidase (1 Behringwerke, Ulm, Germany) was from Calbiochem (La Jolla, CA). Biotinylated anti-rat IgG and ABC Vectastain Kit were obtained from Vector Laboratories (Peterborough, UK). Streptavidin microbeads (1 Behringwerke Ulm, Germany) was from Calbiochem (La Jolla, CA). Bio-rad sheep red blood cells (RBC) were from Flow Laboratories (Irvine, Scotland) and were stored at 4°C and used within 2 weeks.

Cell lines and antibodies. The macrophage-like cell line RAW 264.7 and P388D1 were provided by the Sir William Dunn School of Pathology, Oxford University (Oxford, UK). The Mm1 macrophage-like cell line was provided by Dr T. Masuda (Kyoto University, Kyoto, Japan). NIH 3T3 cells were obtained from the ICRF Cell Bank. The following rat monoclonal antibodies (MoAbs) with specificities shown were used as supernatants or ascites at saturating concentrations: F4/80, specific for mature mouse macrophages; 5C6, which binds CD11b on mouse myeloid cells; 2F8, which recognizes the macrophage scavenger receptor; S7, which recognizes sialoadhesin; 2F8, which recognizes the macrophage scavenger receptor; and ER.HR3 against mouse stromal macrophages.

Separation of Eb and EEN. Eb were enriched from adult mouse bone marrow cells by depletion of myeloid cells. Mice were killed by inhalation of CO2. Bone marrow and bone marrow cells were flushed from both femora and tibiae. Cells were dissociated by pipetting in PBS containing 5 mmol/L EDTA and 1% bovine serum albumin (BSA; PBS/BSA), suspended at 10⁶/mL, and incubated with MoAb 5C6 for 15 minutes on ice. Cells were then incubated for 15 minutes with biotinylated anti-rat IgG (10 μg per 10⁶ cells) in PBS/BSA, followed by 10 minutes with streptavidin magnetic microbeads (100 μL per 10⁶ cells) in PBS plus 5 mmol/L EDTA. Throughout the procedure, cells were kept on ice, and after each incubation they were washed twice with the appropriate buffer. The labeled cell suspension was then passed through a magnetic cell separation column (MACS; Miltenyi Biotech), and nonmagnetic cells were collected. These included the majority of Eb, contaminating BCC, reticulocytes, and B lymphocytes. This cell suspension was layered onto a self-generated 70% Percoll continuous density gradient (500g, 4°C for 20 minutes) and centrifuged at 500g at 4°C for 30 minutes. Eb (greater than 90% pure on May-Grünwald-Giemsa–stained cytopreps) were collected from the lowest density fraction of the gradient, washed twice in PBS, and resuspended in DMEM plus 20% FCS. To obtain and isolate EEN, the purified Eb were cultured at 37°C in a 7% CO₂ atmosphere in DMEM containing 20% FCS and 1.5 mmol/L murine erythropoietin. After 24 hours of culture, the cells were harvested, washed in PBS, and layered onto a self-generated 70% Percoll density gradient as described above. EEN were collected from the highest density fractions of the gradient, washed twice in PBS, and resuspended in assay solution at an appropriate concentration. Cells from different fractions were analyzed by staining cyto-centrifuge smears with May-Grünwald-Giemsa.

Electron microscopy. Eb and EEN were isolated as described above. Cell pellets were fixed in 2.5% (wt/vol) glutaraldehyde in 0.1 mol/L Sorensens phosphate, pH 7.4, for 2 hours at room temperature. After washing (three times for 5 minutes) with Sorensens’s phosphate buffer, the pellets were postfixed in 1% osmium tetroxide in the same buffer for 60 minutes. The samples were processed by dehydration in graded series of ethanol followed by propylene oxide and embedded in araldite resin (Taab Laboratories Equipment Ltd, Reading, UK). Sections were stained for 10 minutes in a 2% (wt/vol) solution of saturated lead citrate and examined with a Jeol 1200 electron microscope (Jeol, Tokyo, Japan).

Isolation of macrophage populations. RBMM were isolated as described previously with some modifications. Briefly, adult femoral and tibial bone marrow plugs were digested with 0.05% collagenase in RPMI 45 to 60 minutes with constant rotation. Hematopoietic clusters enriched for RBMM were separated from single cells by centrifugation at 500 rpm for 10 minutes on a self-generated density gradient of 30% Percoll prepared as described above. Clusters were resuspended in RPMI plus 10% FCS and allowed to adhere to 5-mm diameter, 12-spot glass slides (PH-086; Hendley, Essex, UK) for 3 to 4 hours at 37°C in 5% CO₂. Hematopoietic cells were detached from adherent RBMM by incubation in PBS for 20 minutes followed by flushing with PBS using a wide-bore plastic pipette. Residues of peritoneal macrophages were obtained from untreated mice after lavage of the peritoneal cavity with PBS. Bronchoalveolar macrophages were collected by tracheal cannulation followed by lavage with PBS plus 0.5 mmol/L EDTA. Spleen and mesenteric lymph node macrophages were obtained by mincing tissues from mice and digesting them with 0.05% collagenase in RPMI for 60 minutes. Undigested material was discarded. The macrophage-like cell lines Mn1, J774, and P388D1 were cultured in DMEM plus 10% FCS. Before binding assays, the cells were washed twice in RPMI, resuspended in RPMI plus 10% FCS, and plated onto 12-spot glass slides at 500 to 1,000 adherent cells per spot. Nonadherent cells were removed by gentle, direct pipetting with RPMI after 3 to 4 hours of incubation at 37°C in 5% CO₂.

Binding and phagocytosis assays. Binding assays were performed in media either with (DMEM plus 20 mmol/L HEPES) or without (PBS plus 0.1 mmol/L EDTA) divalent cations. Slides with adherent macrophages were rinsed three times in the assay medium. Cells and nuclei, prepared as described above, were washed three times in PBS and resuspended in assay medium. Forty microliters of the cell suspension (5 × 10⁶/mL of Eb or EEN; 1% vol/vol sheep RBC) were added to adherent macrophages on each untreated slide. Three slides were placed in a humidified chamber and incubated for 45 minutes at 37°C in 5% CO₂. Unbound cells or nuclei were removed by gently rinsing the slides in the assay medium. Cells were then fixed for 15 minutes at room temperature in PBS containing either 4% paraformaldehyde for fluorescence staining or 0.25% (vol/vol) glutaraldehyde for immunohistochemistry and then rinsed twice in PBS. Macrophages were identified morphologically by phase-contrast microscopy or by immunolabeling with F4/80. EEN and Eb were identified by simultaneous Dil and DAPI fluorescence. Spleen RBC were identified by phase-contrast microscopy in binding assays. Macrophages were considered positive if they bound three or more cells. Assays were routinely performed in duplicate, and 150 to 200 macrophages were scored on each spot.

Phagocytosis assays were performed in DMEM plus 20 mmol/L HEPES. At the end of the 45-minute incubation period, nonphagocytosed EEN were removed from macrophages by incubation in 100 μg/mL of trypsin at 37°C for 20 minutes, followed by gentle pipetting. Macrophages were then fixed in 4% paraformaldehyde, rinsed, and mounted in 50% glycerol in PBS. Phagocytosis of EEN was determined by both phase-contrast and fluorescence microscopy. Macrophages were scored as positive if they contained one or more EEN. At least 200 macrophages on duplicate spots were counted for each data point. As a control for phagocytosis, zymosan was dissolved in PBS at 1 μg/mL, boiled for 30 minutes, and washed
twice in PBS before use; approximately 10⁶ particles were added to each spot. **Fluorescent staining.** Double fluorescence was used to identify EEN with an intact plasma membrane. The Eb plasma membrane was labeled before culture with the red fluorescent lipophilic dye, Dil, as described previously, with some modifications. Eb, purified as above, were resuspended in DMEM plus 10% FCS. Cells were then incubated with Dil at 25 μg/mL for 30 minutes at 37°C, washed three times in PBS, and cultured with erythropoietin as described above.

Staining of nuclei with DAPI was performed at the end of the binding assays. After fixation, cells on slides were incubated with 1 μg/mL DAPI in PBS for 10 minutes at 37°C, rinsed twice with PBS, and mounted in 50% glycerol in PBS. The efficiency of staining was checked by fluorescence microscopy.

**Immunohistochemistry.** Cells on slides were fixed for 10 minutes at room temperature in 0.25% glutaraldehyde in PBS and rinsed three times in PBS. Cell surface antigens were detected by immunoperoxidase labeling using the avidin-biotin-peroxidase detection system. Cells were then counterstained with Mayer’s hematoxylin and observed by light microscopy.

**Enzyme treatments.** Enzymes were dissolved in RPMI and added to cells at varying concentrations. Digestion was routinely performed for 60 minutes at 37°C and stopped by addition of FCS to a final concentration of 20%, followed by a further 20-minute incubation at 37°C. Cells were washed three times in the assay medium before binding assays.

**RESULTS**

**Isolation of EEN.** To obtain and isolate EEN, our approach was to (1) purify large numbers of Eb from normal mouse bone marrow, (2) culture them in 20% FCS and erythropoietin to stimulate the terminal stages of differentiation and enucleation, and (3) separate the relatively dense EEN from the remaining Eb in the cultures by density gradient centrifugation. Immunomagnetic depletion with a MACS apparatus was found to be an efficient and rapid way of removing myeloid cells from large numbers (greater than 10⁶) of bone marrow cells. The enriched Eb were further purified to greater than 90% by density gradient centrifugation, during which the more dense B lymphocytes, reticulocytes, and extruded nuclei were removed. Optimal numbers of EEN were obtained after cultivation of Eb for 24 hours in the presence of 1.5 U/mL of erythropoietin. Under these conditions, approximately 10% of Eb underwent enucleation, producing nuclei and reticulocytes. The final step in the purification was the separation of the newly generated EEN from Eb by density gradient centrifugation. This resulted in a fraction that contained approximately 80% free nuclei that, by light microscopy, were small and condensed, staining intensely with Giemsa. The remaining approximately 20% of cells were contaminating reticulocytes and erythroblasts. The overall cell yields from a typical experiment of more than 50 performed are shown in Table 1.

Previous studies with freshly prepared bone marrow suspensions have shown that Eb nuclei are extruded with a narrow rim of plasma membrane. It was, therefore, important to determine ultrastructurally whether the purified nuclei obtained by our procedure were surrounded by a limiting plasma membrane (Figs 1 and 2). By electron microscopy, the final EEN fraction contained 70.9% ± 6.3% (mean ± SD) condensed, pyknotic nuclei that were similar morphologically to extruded nuclei obtained in other studies using fresh hematopoietic tissue. However, these nuclei were of two types. In a typical experiment, 63% had a clear rim of plasma membrane and represented authentic EEN, and the remaining 37% were bare nuclei, lacking a plasma membrane (Fig 2). It is likely that the bare nuclei were produced by mechanical disruption during cell separation.

As only the EEN are involved in recognition and phagocytosis by macrophages under physiologic conditions, it was important to distinguish them unambiguously from bare nuclei in subsequent binding assays. To achieve this, we labeled the Eb plasma membrane before culture with the lipophilic red fluorescent dye Dil, and nuclei were stained after binding to macrophages with DAPI. The EEN, but not the bare nuclei, acquire the fluorescent label from the Eb plasma membrane during enucleation. In comparative experiments, labeling Eb with Dil had no effect on the quantity and quality of EEN obtained after cultivation in erythropoietin (data not shown). This is in keeping with other reports that Dil is biologically inert after membrane labeling. Approximately 70% of the nuclei exhibited surface fluorescence after Percoll density purification. This is in agreement with data obtained by electron microscopy, in which 63% of nuclei had intact plasma membranes (see above).

**EEN bind avidly to RBMM.** The macrophage population that naturally binds and phagocytoses EEN is the resident macrophage network of the bone marrow. These cells were, therefore, used in all experiments to characterize the nature of the interaction with EEN. RBMM are isolated as clusters containing a central macrophage and large numbers of attached hematopoietic cells. After attachment of clusters to glass and stripping of the attached cells, approximately 50% of the adherent cells are well-spread stromal macrophages. When these cells were incubated with EEN suspensions, EEN bound rapidly to the majority of RBMM, reaching maximal levels of binding by 45 minutes of incubation. The presence of a red rim of surface fluorescence, together with a condensed nuclear morphology visualized by blue DAPI fluorescence, allowed rapid identification of bound EEN by fluorescence microscopy (Fig 3). Dil-labeled EEN had no effect on the level of binding, and EEN prepared in this way were used routinely in all assays. Because of the existence of two types of nuclei in EEN preparations, we quantified the types of nuclei bound to RBMM and found that an average of 83% ± 8% (mean ± SD) were authentic EEN. The level and specificity of EEN binding were highly reproducible. In more than 40 experiments, approximately

**Table 1. Purification of EEN From Mouse Bone Marrow**

<table>
<thead>
<tr>
<th>Stage of Purification</th>
<th>Nucleated Cell Yield</th>
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<tr>
<td>Total bone marrow</td>
<td>2.1 × 10⁶</td>
</tr>
<tr>
<td>Myeloid-depleted cells</td>
<td>9 × 10⁷</td>
</tr>
<tr>
<td>Percoll-purified Eb</td>
<td>6.5 × 10⁷</td>
</tr>
<tr>
<td>Percoll-purified EEN</td>
<td>5.6 × 10⁷</td>
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Bone marrow cells were flushed from both femora and tibiae from seven Balb/c male mice. Results are from a representative experiment of more than 50 performed.
Fig 1. Purification and culture of erythroblasts. Electron micrographs showing purified Eb and an Eb in the process of extruding its nucleus. Eb were purified from normal mouse bone marrow by depletion of myeloid cells and density gradient centrifugation. Enucleation of purified Eb was stimulated by cultivation in erythropoietin. The purified Eb (A and B) comprise a homogeneous population with a few small mitochondria and some vesicular elements in the cytoplasm. (C) Nuclear extrusion in a single erythroblast; the nucleus gradually moves toward the cell surface and becomes extruded together with a thin rim of cytoplasm.

80% of RBMM bound 3 to 50 EEN, whereas no binding was seen to any other adherent cells, including immature monocytes and neutrophils, fibroblastoid cells, and osteoclasts. This suggests that EENR is a macrophage-restricted receptor.

RBMM uses a novel recognition mechanism to bind EEN. To determine whether the EENR is a surface protein, RBMM were pretreated with trypsin. Binding of EEN to trypsin-treated RBMM was inhibited in a dose-dependent fashion (Fig 4). In contrast, when EEN preparations were pretreated with trypsin, we did not see any inhibitory effect, even at the highest concentration tested (Fig 4). These results indicate that EENR is a trypsin-sensitive cell surface protein that recognizes a trypsin-resistant ligand on EEN.

Binding of EEN to RBMM was independent of temperature, occurring equally at 4°C or 37°C, and was unaffected in the presence of sodium azide, a metabolic inhibitor, and cytochalasin B, which disrupts actin microfilaments of the cytoskeleton. Binding was also independent of divalent cations, being unaltered in calcium- and magnesium-free PBS containing 0.1 mmol/L EDTA (Fig 5A). EEN adhesion to RBMM is, therefore, not dependent on the EbR, a receptor that mediates divalent cation-dependent adhesion of Eb to stromal macrophages (Fig 5A). This also indicates that integrins, C-type lectins, and other divalent cation-dependent mechanisms are not required for EEN binding to macrophages.

Sialoadhesin was first identified on RBMM as a divalent cation-independent receptor that binds unopsonized sheep RBC via recognition of sialylated glycoconjugates. EENR is distinct from sialoadhesin because SER-4, a blocking MoAb against sialoadhesin, had no inhibitory effect on binding of EEN at 20 μg/mL, while it was able to completely block binding of sheep RBC to RBMM at the same concentration (Fig 5B). In addition, pretreatment of EEN with sialidase had no effect on binding to RBMM, whereas parallel treatment of sheep RBC eliminated binding (data not shown).

Some macrophage populations express the scavenger receptor, which is capable of recognizing a wide array of different ligands. Experiments with an inhibitory anti-mouse macrophage scavenger receptor MoAb, 2F8, showed that the binding of EEN to RBMM is unlikely to be mediated by this receptor (Table 2). This conclusion was consistent with the failure of RBMM to endocytose Dil-labeled acetylated-low-density lipoprotein (LDL), a ligand for the scavenger receptor (data not shown). Finally, a variety of other MoAbs
that bind mouse macrophages were found to have no effect on binding of EEN to RBMM (Table 2).

To explore the possibility that recognition of EEN involves lectin-like interactions, we examined the effect of various monosaccharides (Table 2). These included the amino sugars D-glucosamine, D-galactosamine, and D-mannosamine, which have previously been shown to block macrophage recognition of apoptotic neutrophils. 60 None of the

**Fig 2.** Electron micrographs of purified EEN. EEN were obtained by cultivation of Eb in the presence of erythropoietin followed by density gradient centrifugation. The purified EEN show condensed nuclei, some of which are bare nuclei lacking an intact membrane (A and B), and others have intact plasma membranes and represent authentic EEN (C and D). Nuclear envelopes are identified in authentic EEN (arrows).

**Fig 3.** Binding of EEN to RBMM. Eb were labeled with Dil and cultured with erythropoietin, and the EEN were purified and incubated with RBMM for 45 minutes at 37°C. Slides were washed and fixed in paraformaldehyde, and nuclei were labeled with DAPI. The photomicrographs (A-C) show a single well-spread RBMM to which nuclei are bound. DAPI specifically stains the nuclei, most of which are small and condensed (B). Staining with Dil (C) showed which of the nuclei had retained plasma membrane and, therefore, represent EEN. Bar represents 10 μm.
sugars tested had any effect on EEN binding, even at concentrations up to 100 mmol/L.

Macrophages express a receptor for phosphatidylserine that is involved in recognition of certain tumor cells and apoptotic thymocytes. This receptor has not been characterized at a molecular level, but it can be blocked by glycerophosphoryl-L-serine and phospho-L-serine. Use of these compounds at concentrations up to 10 mmol/L had no effect on binding of EEN to RBMM (Table 2), again suggesting that the EENR is distinct from previously described macrophage receptors.

**Distribution of EENR on various macrophage populations.** A variety of different macrophage populations were isolated to determine their expression of EENR. As shown in Table 3, 65% of F4/80-positive adherent cells from spleen showed positive binding; this was most evident on the large, well-spread macrophages compared with smaller, immature cells. With mesenteric lymph node macrophages, binding was again only observed on the large, well-spread macrophages, which constituted approximately 20% of the F4/80-labeled cells. The most striking binding was seen with resident peritoneal macrophages, greater than 90% of which showed intense rosetting, even higher than that seen with RBMM (Table 3). In contrast, alveolar macrophages showed no binding whatsoever. The binding of EEN to peritoneal cavity macrophages was independent of divalent cations and was identical to that of RBMM by all criteria tested (data not shown). These results suggest that EENR is expressed differentially by both stromal tissue macrophages and peritoneal cavity macrophages. We also investigated the expression of EENR on the macrophage-like cell lines Mm1, RAW 264.8, and P388D1 and the fibroblastic cell line NIH3T3. Binding of EEN was observed to all macrophage-like cell lines but was restricted to the large, firmly adherent, and well-spread population. Virtually no binding of EEN was observed with NIH3T3 cells.

**Phorbol myristate acetate (PMA) induces macrophages to phagocytose EEN.** To determine whether the binding of EEN to macrophages was accompanied by phagocytosis, bound EEN were removed from the macrophages by treatment with trypsin and gentle pipetting. Under the usual conditions for binding assays (45 minutes, no serum), fewer than 10% of RBMM were seen to phagocytose EEN. This low level of phagocytosis was increased to 20% in the presence of 10% serum, but higher levels were not observed, even after 24 hours of culture. The failure of RBMM to phagocytose EEN was not due to a general defect in phagocytic activity, as large numbers of unopsonized zymosan particles were phagocytosed by greater than 90% of the macrophages when incubated under identical conditions (data not shown).

Previous studies on complement receptors have shown...
that PMA is able to trigger phagocytosis in unstimulated macrophages. Therefore, we performed experiments to determine whether PMA could stimulate uptake of EEN (Figs 6 and 7). When RBMM were pretreated with PMA for 30 minutes before the binding assay, a dose-dependent increase in the percentage of RBMM that ingested EEN was seen (Fig 6). An effect of PMA was observed at the lowest concentration tested, 10 ng/mL, and at 1 μg/mL, the highest concentration, approximately 70% of macrophages contained 1 to 10 EEN (Figs 6 and 7). The ability of PMA to induce phagocytosis of EEN was not enhanced when macrophages were pretreated for various periods (data not shown).

Attempts to stimulate phagocytosis by adherence of macrophages to fibronectin-coated glass were unsuccessful (data not shown).

DISCUSSION

In this study we have developed a method to produce and isolate EEN in vitro and have characterized the nature of their interaction with RBMM. Our observations provide evidence for the existence of a novel divalent cation-independent macrophage receptor that binds avidly to EEN and, under appropriate circumstances, can lead to their phagocytosis. We suggest that this receptor is involved in recognition and phagocytosis of EEN within hematopoietic tissues.

As the nuclei extruded from Eb are rapidly digested by macrophages, it is difficult to isolate EEN directly from hematopoietic tissues. However, previous work demonstrated that when Eb were cultured in the presence of a physiologic concentration of erythropoietin, they could undergo terminal differentiation to yield nuclei and reticulocytes in a manner very similar to that seen in vivo. In agreement with that study, we obtained and isolated a workable number of EEN from erythropoietin-stimulated Eb in vitro. Because mechanical disruption of membranes may occur during cell separation, Eb nuclei may be released from lysed cells in a different manner from the naturally occurring enucleation process, in which nuclei are extruded with an intact rim of plasma membrane. This distinction was observed in the present study during ultrastructural analysis of isolated Eb nuclei in which two types of free nuclei could be distinguished. EEN with an intact plasma membrane were contaminated by bare nuclei without a plasma membrane. To distinguish extruded from bare nuclei in subsequent experiments, we labeled the

Table 2. Effect of Various Reagents on Binding of EEN to RBMM

<table>
<thead>
<tr>
<th>Reagent</th>
<th>% Control Binding</th>
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<tbody>
<tr>
<td>MoAb/Specificity</td>
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<tr>
<td>2F8/scavenger receptor</td>
<td>97.9 ± 1</td>
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<tr>
<td>S7 + S11 + S18/leukosialin (CD43)</td>
<td>108.3 ± 5</td>
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<tr>
<td>FA11/macrosialin (CD68)</td>
<td>112.1 ± 2.3</td>
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<td>5C8/Mac-1 (CD11b)</td>
<td>100.2 ± 2</td>
</tr>
<tr>
<td>F4/80/macrophage antigen</td>
<td>98.9 ± 4</td>
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<tr>
<td>ER.HR3/macrophage antigen</td>
<td>98.2 ± 5.2</td>
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Monosaccharide

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<tr>
<th>Monosaccharide</th>
<th>% Control Binding</th>
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<tr>
<td>D-glucose</td>
<td>100.6 ± 9.7</td>
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<tr>
<td>D-galactose</td>
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<tr>
<td>D-fucose</td>
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<tr>
<td>D-glucosamine</td>
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<tr>
<td>D-galactosamine</td>
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<td>D-mannosamine</td>
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<tr>
<td>N-acetyl-D-glucosamine</td>
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<tr>
<td>N-acetyl-D-galactosamine</td>
<td>97.2 ± 6.2</td>
</tr>
<tr>
<td>N-acetyl neuraminic acid</td>
<td>97.7 ± 0.7</td>
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Others

<table>
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<tr>
<th>Others</th>
<th>% Control Binding</th>
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<tr>
<td>Glycerophosphoryl-L-serine</td>
<td>97.7 ± 2.5</td>
</tr>
<tr>
<td>Phospho-L-serine</td>
<td>98.8 ± 5.3</td>
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</table>

Binding assays were performed in the presence of MoAbs at saturating concentrations, monosaccharides at 100 mmol/L, glycerophosphoryl-L-serine at 10 mmol/L, and phospho-L-serine at 10 mmol/L. Values show mean ± 1 SD of data pooled from three experiments.

Fig. 6. Effect of PMA on the induction of phagocytosis of EEN by macrophages. Binding assays were performed in DMEM either with or without FCS in the presence of the indicated amounts of PMA. Data show the means ± 1 SD from two experiments.

Table 3. Binding of EEN to Tissue Macrophages and Macrophage-Like Cell Lines

<table>
<thead>
<tr>
<th>Macrophage Population or Cell Line</th>
<th>% Cells Binding ≥ 3 EEN</th>
</tr>
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<tbody>
<tr>
<td>Bone marrow</td>
<td>78.6 ± 5.3</td>
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<tr>
<td>Spleen</td>
<td>64.8 ± 9.7</td>
</tr>
<tr>
<td>Lymph node</td>
<td>19.0 ± 10.3</td>
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<tr>
<td>Peritoneal cavity</td>
<td>89.8 ± 4.3</td>
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<tr>
<td>Alveolar</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>RAW 264.7</td>
<td>7.4 ± 0.7</td>
</tr>
<tr>
<td>Mm1</td>
<td>3.7 ± 2.3</td>
</tr>
<tr>
<td>P388 D1</td>
<td>21.0 ± 8.3</td>
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<tr>
<td>NIH 3T3</td>
<td>0.25 ± 0.35</td>
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Tissue macrophages were identified by immunohistochemistry using MoAb F4/80, and only these cells were included in the analysis. F4/80-negative cells did not bind EEN. Values show mean ± 1 SD of data pooled from three experiments.
Eb plasma membrane with a fluorescent dye, DiI, before culture. The EEN could then be identified by the presence of a weak but clear rim of red DiI fluorescence.

The susceptibility of EENR to treatment with trypsin shows that it is a cell-surface protein. However, several lines of evidence indicate that the binding of EEN to RBMM is not mediated by a previously characterized macrophage receptor. The pattern of expression of EENR is distinct from both EbR and sialoadhesin, which are expressed on the majority of stromal macrophages but not on normal peritoneal macrophages. In addition, EbR is a divalent cation-dependent receptor, and sialoadhesin-mediated binding is sensitive to sialidase treatment of ligand-bearing cells. It was also shown in the present study that the binding of EEN to RBMM is not inhibited by a sialoadhesin-specific blocking MoAb. EENR is distinct from the macrophage scavenger receptor as evidenced by its specific tissue distribution and the failure of a scavenger receptor-specific MoAb to block its activity.

Previous studies demonstrated a reduced negative charge on the plasma membrane surrounding the extruded nucleus compared with that of the reticulocyte. It was, therefore, assumed that this may be a mechanism by which macrophages recognize EEN as particles destined for phagocytosis. Our studies suggest, however, that the interaction is not mediated simply by a charge effect, because N-acetyl neuraminic acid and several cationic sugars did not inhibit binding, even up to a concentration of 100 mmol/L.

Macrophage recognition and ingestion of EEN is a swift, efficient way of removing effete nuclei from hematopoietic tissues without the release of potentially toxic substances that might damage neighboring cells. This function is similar to that of macrophage recognition and removal of apoptotic cells, a process that is believed to involve at least three recognition pathways. Depending on the cell type, the surface exposure of thrombospondin, specific carbohydrate, or phosphatidylserine may lead to macrophage recognition and phagocytosis via the vitronectin receptor and CD36, lectin-like receptors, and a phosphatidylserine receptor, respectively. As binding to the EENR was divalent cation-independent, integrins like the vitronectin receptor and C-type lectins such as the mannose receptor are unlikely to play a dominant role. In addition, amino sugars that block the thrombospondin-dependent recognition of apoptotic neutrophils had no effect on EEN binding, nor did phosphoglycerocerase, which blocks uptake of apoptotic thymocytes through the phosphatidylserine receptor. Taken together, our results argue that the EENR is a novel macrophage receptor.

Despite the dissimilarities of EENR to previously characterized macrophage receptors involved in recognition of apoptotic cells, it is possible that EENR is involved in this process as well as others, such as recognition of senescent RBC. In the bone marrow, for example, RBMM are believed to ingest large numbers of apoptosing B cells, but the receptors and ligands involved in their specific recognition have not yet been characterized. In addition, the high expression of EENR on peritoneal cavity macrophages suggests that this receptor is involved in functions other than recognition of EEN.

Previous studies have demonstrated that as EEN are extruded, they are rapidly phagocytosed by macrophages both in vivo and in long-term bone marrow cultures. Some investigators have estimated that up to 40 EEN may be phagocytosed by a single macrophage in a day. However, under our initial experimental conditions, we did not observe significant RBMM phagocytosis of EEN for culture periods of up to 24 hours. Control experiments with zymosan showed that the macrophages used in this study had a normal capacity for phagocytosis. Our original explanation for this disparity was that phagocytosis of EEN in vivo may be more complicated, perhaps requiring an extra inducing factor or specific stromal components that are not present in vitro. We were, therefore, interested in searching for compounds or conditions that stimulate phagocytosis of EEN. Previous studies showed that both C3b and C3b' receptors on human
monocytes efficiently mediate adherence of RBC coated with the corresponding ligands but do not promote their ingestion. However, these cells become capable of ingesting C3-coated RBC after treatment with PMA.\textsuperscript{33,34} These findings prompted us to test whether PMA was similarly capable of stimulating the phagocytosis of EEN by RBMM. Indeed, the capacity of RBMM to phagocytose EEN increased in a dose-dependent fashion in the presence of PMA. These findings establish the important principle that RBMM are capable of phagocytosing EEN in vitro and that this can be regulated. However, in the present experiments, it was not clear whether the effect of PMA is specific for EENR or only reflects a general enhancement of cellular phagocytic capacities. The fact that only a relatively small proportion of EEN that bound to RBMM were phagocytosed, even at high concentrations of PMA, suggests that specific components of the microenvironment may be required for efficient phagocytosis of EEN in vivo.

The expression of EENR is restricted to mature macrophages; no binding of EEN to contaminating monocytes or neutrophils in macrophage preparations was observed. The heterogeneity of EENR distribution between various macrophage populations may reflect the effects of local and systemic regulation on EENR expression. Due to lack of specific MoAbs or inhibitors of EbR and EENR, we have been unable to determine whether, in the presence of divalent cations, the binding of EEN to RBMM can be mediated by EbR as well as EENR. We have not characterized the EENR ligands and, therefore, cannot determine whether preexisting ligands become modified or whether EEN acquire a novel ligand(s) during the enucleation process. Previous studies demonstrated that glycoconjugates recognized by Con A were enriched in the EEN plasma membrane during enucleation and that this process was accompanied by a reduction in the negative charge on the surface of EEN.\textsuperscript{5,10,11} It is possible that molecular remodeling of the EEN plasma membrane results in two important events with respect to macrophage recognition. First, if potential ligands for EENR become concentrated on the EEN plasma membrane during enucleation, this could trigger binding and phagocytosis of EEN. Second, concomitant depletion of ligands for EbR from the reticulocyte membrane could promote detachment of reticulocytes from the RBMM surface.

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Extruded erythroblast nuclei are bound and phagocytosed by a novel macrophage receptor

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