PROPERTIES AND DISTRIBUTION OF A LECTIN-LIKE HEMAGGLUTININ DIFFERENTIALLY EXPRESSED BY MURINE STROMAL TISSUE MACROPHAGES

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Resident macrophages (Mϕ)[1] are widely distributed throughout tissues of adult animals, even in the absence of overt inflammation or infection. At portals of entry they may play a role in innate resistance to infection, but in other sites, particularly hematopoietic and lymphoid tissues where stromal Mϕ are relatively abundant (1–3), their precise functions are less clear. It is possible, however, that in addition to scavenging worn-out and damaged cells and ingesting erythrocyte nuclei, stromal Mϕ in these tissues may play a regulatory role in cellular proliferation and differentiation. Support for this possibility has come from studies on bone marrow and thymus (2–5). In both cases, stromal Mϕ establish intimate physical associations in situ with immature hematopoietic cells or thymocytes (2, 3). After enzymatic dispersion of the tissues, it is possible to obtain clusters that contain Mϕ and attached cells (4, 5). To date, there have been few attempts to determine the functional importance of cluster formation in these tissues or to characterize the receptors and ligands involved. To carry out such studies, it is necessary to isolate the relevant Mϕ, rather than to use a more easily obtained population such as peritoneal Mϕ, which may differ substantially in function and properties (5).

We recently described (5) a method for isolating resident Mϕ from murine bone marrow (RBMM), in reasonable yield and purity, which allowed us to study the properties of RBMM (5). When their phenotype was compared with that of resident peritoneal Mϕ (RPM), we found both quantitative and qualitative differences in expression of various antigens and receptors. The most striking difference was the ability of RBMM, but not RPM, to bind unopsonized sheep E without ingestion. In view of the possibility that this novel E receptor (SER) could be involved in Mϕ-binding to hematopoietic cells in murine bone marrow, it seemed important to characterize it further. In the present paper we show that SER on RBMM is a lectin-like agglutinin with specificity for sialylated component(s) on E. Our studies show that SER is also expressed on stromal Mϕ isolated

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[1] *Abbreviations used in this paper:* EIgG, E opsonized with rabbit anti-E antibody; MFR, mannosyl-fucosyl receptor; Mϕ, macrophage(s); NANA-lactose, *N*-acetylneuraminyllactose; RBMM, resident bone marrow Mϕ; RPM, resident peritoneal Mϕ; SER, sheep erythrocyte receptor.

from lymph node, liver, and spleen, but is at low levels or undetectable on monocytes and Mϕ isolated from thymus, peritoneal cavity, pleural cavity, and bronchoalveolar spaces. The presence of SER on selected stromal but not "free" Mϕ populations suggests that this hemagglutinin may be involved in adhesive interactions within tissues.

Materials and Methods

Animals. Mice were bred at the Sir William Dunn School of Pathology, University of Oxford and both sexes were used between 8 and 12 wk of age.

Media and Reagents. RPMI 1640 was obtained from Gibco-Biocult Ltd., Paisley, United Kingdom. FCS was obtained from Seralab UK Ltd., Crawley Down, United Kingdom, and was routinely heat inactivated for 30 min at 56°C. Media were supplemented with glutamine (2 mM) and gentamicin (20 μg/ml). PBS without calcium or magnesium was obtained from Oxoid Ltd., Basingstoke, United Kingdom. Hepes buffer was purchased from Gibco-Biocult Ltd. Collagenase was purchased from Boehringer Corp., Lewes, United Kingdom; and DNase (type I), trypsin (type IX), and *Staphylococcus aureus* protease (type XVII) were from Sigma Chemicals Ltd., Poole, United Kingdom. Neuraminidase (*Vibrio cholerae*) was obtained from BDH Ltd., Poole, England (500 U/ml), or from Calbiochem-Behring Corp., La Jolla, Ca. (1 Behringwerke unit/ml). Mono- and disaccharides were purchased from Sigma Chemicals Ltd. The trisaccharide, *N*-acetylneuraminyllactose (NANA-lactose) was obtained from either Sigma Chemicals Ltd. or from Boehringer Corp. From Sigma Chemicals Ltd. we purchased NANA-lactose purified from either bovine colostrum (85% 2,3-Neu-5-AcLac) or from human colostrum (85% 2,6-Neu-5-AcLac). The bovine form was also purchased from Boehringer Corp. The gangliosides GM1 and GD1a, purified from bovine brain, were generously provided by Dr. J. Mellanby, Department of Experimental Psychology, Oxford University. The neoglycoconjugates, mannosylated BSA, and galactosylated BSA, with 33–37 moles of sugar per mole protein, were kind gifts of Dr. P. Stahl, Washington University, St. Louis, MO. Ovine submaxillary mucin, prepared according to Hill et al. (6), and ovine orosomucoid (Sigma Chemicals Ltd.) were generously provided by Dr. K. McCusker, Washington University, St. Louis, MO. Fetuin (type III) was purchased from Sigma Chemicals Ltd. Sodium azide and the disodium salt of tetraacetic acid EDTA were from Sigma Chemicals Ltd. Sodium iodoacetate was from BDH Ltd. Sheep E, in Alsevers solution, were purchased from Gibco Biocult Ltd., stored at 4°C, and used within 2 wk.

Antibodies. The following mAbs were obtained as shown and used at saturation as concentrated supernatants: F4/80, a rat mAb specific for mature mouse Mϕ (7); M1/70, which binds to Mac-1, an epitope of the iC3b receptor (CR3) on mouse neutrophils, Mϕ and NK cells (8; Dr. T. Springer, Harvard Medical School, Boston, MA); M5/114, a rat anti-mouse mAb that recognizes Ia antigens ($I\text{-}A^{b,d,q}I\text{-}E^{d,k}$) (9; Dr. H. Waldmann, Department of Pathology, University of Cambridge, United Kingdom); and 2.4G2 a rat mAb directed to the trypsin resistant FcR for IgG1/2b isotypes (10; Dr. J. Unkeless, The Rockefeller University, New York).

Isolation of Mϕ Populations. RBMM were isolated from mouse femoral marrow plugs as described previously (5). Briefly, plugs from 12 femora were digested with 0.05% collagenase and 0.001% DNase dissolved in RPMI. The resulting clusters, containing RBMM, were purified from single cells by velocity sedimentation and were allowed to adhere to 24 × 11 mm diameter coverslips for 2–3 h at 37°C, 5% CO_2, in a fully humidified incubator. RBMM were then stripped free of clustering cells by prior incubation in PBS followed by gentle direct flushing.

All peritoneal Mϕ populations were obtained by lavage with 6 ml of PBS. RPM were harvested from unstimulated mice, inflammatory peritoneal Mϕ were from mice injected intraperitoneally 4–5 d previously with 1 ml of Brewers thioglycollate broth (Difco, E. Mosely, United Kingdom), and immunologically activated peritoneal Mϕ were obtained from mice injected intraperitoneally 12 d previously with either 10^7 live Bacillus Calmette-Guerin BCG organisms (Pasteur strain 1011; kindly provided by Dr. R. North, Trudeau

Institute, Saranac Lake, NY) or killed *Corynebacterium parvum* (Wellcome Biotechnology Ltd., Beckenham, United Kingdom).

Resident pleural Mϕ were obtained from unstimulated mice by pleural lavage with 2 ml of PBS. Bronchoalveolar Mϕ were obtained by tracheal cannulation followed by lavage with 5 × 1-ml aliquots of PBS. Peritoneal, pleural, and bronchoalveolar Mϕ were washed once in PBS and plated onto 11-mm glass coverslips at a density of 10^5 cells per coverslip in 1 ml RPMI plus 10% FCS. After 60 min at 37°C, nonadherent cells were removed by gentle, direct pipetting with RPMI. The adherent cells were then cultured in RPMI plus 10% FCS before rosetting assays.

Spleen adherent cells were obtained from collagenase-perfused spleens as described (11). The cell suspension from one spleen was distributed among eight coverslips, and after 2–3 h at 37°C, nonadherent cells were removed by direct pipetting. Kupffer cells were obtained from collagenase-perfused livers as described (11). The nonparenchymal cell fraction from each liver was plated onto eight coverslips in RPMI plus 10% FCS, and nonadherent cells were removed by pipetting after 30 min at 37°C to minimize contamination by endothelial cells. Thymic and mesenteric lymph node–adherent cells were obtained by mincing tissues from one mouse in 20 ml RPMI plus 0.05% collagenase and 0.001% DNase, and digesting them at 37°C for 60 min with constant rotation at 25 rpm. on a 17-cm diameter rotary wheel. Undigested material was discarded and cell suspensions were washed twice in RPMI by centrifugation for 10 min at 200 *g*. The cells were resuspended in RPMI plus 10% FCS and plated onto four coverslips. Nonadherent cells were removed after 2 h at 37°C.

Isolation of Monocytes. Mice were killed with CO_2 and bled by cardiac puncture using 10% acid citrate dextrose or 10 U/ml heparin (Flow Laboratories, Irvine, United Kingdom) as anticoagulants. Buffy coat, obtained by centrifugation of blood at 100 *g* for 15 min, was suspended in 2 ml PBS and layered onto 8 ml of Lympholyte M (Sera-Lab Ltd., Crawley Down, United Kingdom). After centrifugation for 40 min at 400 *g*, the mononuclear cells at the interface were collected and washed four times at 200 *g* for 10 min to remove platelets. Cells were finally suspended in RPMI plus 10% FCS at 10^6 cells per ml and 1-ml aliquots added to coverslips. After 60 min at 37°C, nonadherent cells were removed by repeated, direct pipetting.

Opsonization of E with IgG. E were washed four times in PBS by centrifugation at 500 *g* for 5 min, were resuspended at 5% vol/vol in RPMI plus 1% FCS and incubated for 30 min at 37°C with a subagglutinating concentration of rabbit anti–E IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands). Opsonized E (EIgG) were then washed four times in PBS before use.

E and EIgG Rosetting Assays. Coverslips with adherent cells were rinsed three times by immersion in RPMI and placed in 0.5 ml RPMI, with or without inhibitors, in a 24-well tissue culture dish. EIgG or E, washed four times with PBS by centrifugation for 5 min at 400 *g*, were resuspended in RPMI at 5% vol/vol and 50 μl of the suspension added to the coverslips and were mixed by swirling. After 30 min at 37°C (or the temperature under test), unbound E or EIgG were removed by immersing coverslips three times in each of four beakers of RPMI. In between each beaker the excess fluid on coverslips was drained by touching the edge on tissue paper. After the final rinse, coverslips were placed in 1 ml of 1% vol/vol glutaraldehyde in PBS and stored at 4°C until examination by phase-contrast microscopy. In some experiments, ingestion of E was assessed after lysing bound E by immersing coverslips in 10 mM phosphate buffer (pH 7.2) for 10 s. For immunocytochemistry (see below) cells were fixed in 0.25% glutaraldehyde for 10 min at room temperature and then rinsed three times in PBS. Assays were routinely carried out in duplicate; a cell was considered positive for SER if ≥5 E were attached. Usually, 200 MØ were scored on each coverslip.

In some experiments, rosetting was carried out in suspension as follows: splenic or bone marrow cells and clusters obtained by collagenase digestion were incubated at 4°C in PBS containing 5 mM glucose, 0.5 mM EDTA and were gently dispersed into single cells by passage through a 27-gauge needle. The cell suspension was adjusted to 5 × 10^6 cells/ml and washed E was added at a ratio of 10 E per nucleated cell. After thorough mixing, the

suspension was centrifuged at 200 *g* for 15 min. The pellet was gently resuspended in 5 ml PBS containing 0.5 mM EDTA and the proportion of rosetted cells (≥5 E) was assessed by phase-contrast microscopy. For further analysis of rosettes, unbound E were removed by velocity sedimentation; 10^7 nucleated cells in 5 ml PBS/EDTA were layered over 40 ml PBS containing 0.5 mM EDTA, 5 mM glucose, 3% BSA, and 20 mM Hepes, pH 7.3, in a 50-ml syringe. After 1 h at room temperature, 5-ml fractions were collected from the bottom of the syringe and inspected by phase-contrast microscopy. Fractions free of unbound E were pooled, adjusted to 10^6 nucleated cells/ml, and cytocentrifuge preparations were made from 1-ml aliquots. Slides were fixed "wet" in 0.25% glutaraldehyde and processed for immunocytochemistry.

Immunocytochemistry. Cell surface antigens on adherent and cytocentrifuge preparations were detected by immunoperoxidase labeling, using an avidin-biotin detection system as described (5).

Enzyme Treatment. Enzymes were added to or dissolved in RPMI as required and 0.5-ml aliquots were added to Mϕ on coverslips prewashed with RPMI, or to 25 μl of washed, packed E. After mixing, cells were incubated for 60 min at 37°C and digestion was ended by adding 0.5 ml RPMI + 20% FCS. Coverslips were rinsed nine times by immersion in RPMI; E were washed three times in PBS by centrifugation.

Assays Measuring Inhibition by Various Reagents. Sugars, neoglycoproteins, sialoglycoproteins, metabolic inhibitors, and EDTA were dissolved at various concentrations in RPMI. Where necessary, the pH was adjusted to the original value with 0.1 M NaOH, and 0.5-ml aliquots were added to RBMM on coverslips prewashed with RPMI. E-rosetting assays were carried out after 15 min preincubation at 37°C in the continued presence of inhibitors.

Results

Resident Bone Marrow Mϕ Express a Novel E Receptor. RBMM were purified from hematopoietic cell clusters by selective adherence to glass and removal of attached cells, after collagenase digestion of femoral bone marrow, as described (5). Two classes of adherent cells were obtained by this procedure. ~50% were large, well-spread cells of which >90% were RBMM, staining strongly with the Mϕ-specific mAb F4/80 (7). The remaining 50% were small immature neutrophils and monocytes, the latter staining weakly with F4/80. Other adherent cells, present at ~1% of the total, included large, well-spread osteoclasts and fibroblast-like cells. When these preparations were incubated with unopsonized E, the majority of RBMM, but none of the other adherent cells, rapidly formed rosettes (Fig. 1*A*). In comparison, rosette formation to RPM was rarely observed (Fig. 1*B*). Binding of E to RBMM was maximal by 30 min of incubation and was not accompanied by significant ingestion (not shown). This phenomenon was highly reproducible and was not due to the collagenase or DNase used for their isolation, since the few RBMM that could be obtained without enzymes rosetted indistinguishably and treatment of RPM with collagenase and DNase did not reveal a "cryptic" form of SER. To examine the fate and stability of bound E, rosettes were cultivated for varying periods up to 24 h in RPMI or RPMI + 10% FCS. At all time points examined we did not observe significant ingestion. Instead, E slowly detached at a rate of ~5% per hour in RPMI and ~10% per hour in RPMI + 10% FCS (not shown).

Arbitrarily, positive rosetting in all experiments was defined by the ability to bind five or more E. However, the majority of positive RBMM bound >20 E and in 30 independent experiments we found that an average of 70% ± 15 (mean ± 1 SD; range, 29–91%) bound ≥ 5 E. The variability in rosette formation

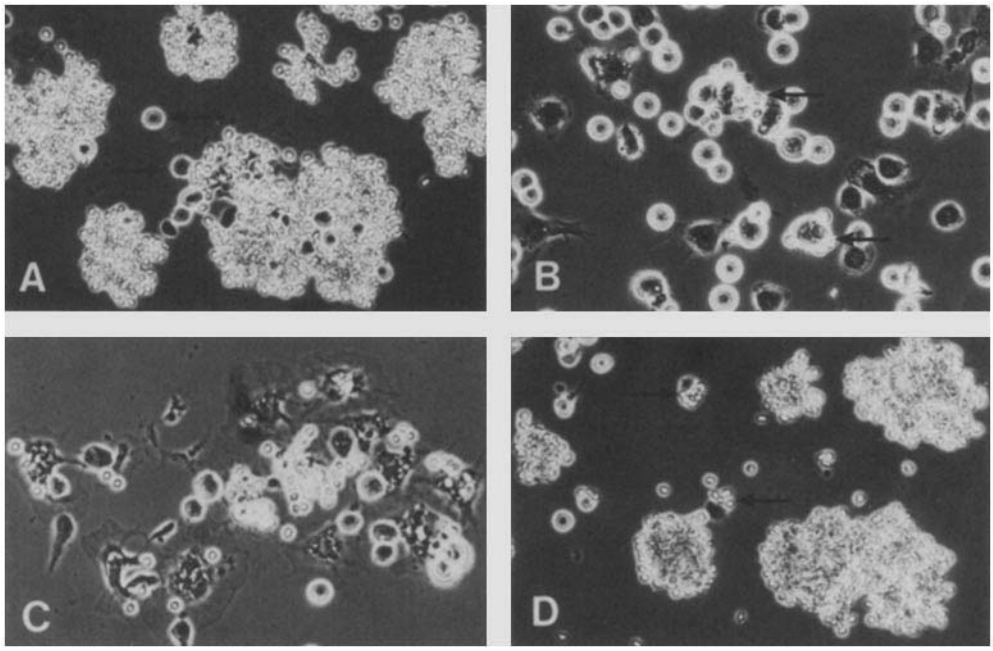

FIGURE 1. RBMM express a novel E receptor. Phase contrast micrographs of rosetting assays with RBMM (*A*,*C*, and *D*) and RPM (*B*) after adherence and purification on glass coverslips (× 200). Mϕ were incubated with a 0.5% vol/vol suspension of E or EIgG in RPMI for 30 min at 37°C. Unbound erythrocytes were removed by repeated immersion of coverslips in RPMI and cells were fixed in 1% glutaraldehyde before examination. (*A*) Binding of E to RBMM but not to contaminating monocytes and neutrophils (*arrows*). (*B*) Low binding of E to RPM with only occasional rosettes (*arrows*). (*C*) Inhibition of E binding to RBMM by 20 mM NANA-lactose. Coverslips were preincubated in NANA-lactose for 15 min at 37°C before addition of E. Same experiment as shown in *A*. (*D*) Binding of EIgG to RBMM in presence of 20 mM NANA-lactose, as described in (*C*). Monocytes and neutrophils also form rosettes with EIgG (*arrows*), unlike with E. Same experiment as in *A* and *C*.

seen between experiments was not due to the different batches of E. In addition, the age (6–12 wk) and sex of mice did not appear to affect the ability of RBMM to form rosettes. To determine whether SER was present among other inbred mouse strains, RBMM were isolated from ASN, CBA T6T6, and BALB/c mice. There were no significant differences in expression of SER and in all cases, ingestion of rosetted E was <1% of those bound.

To confirm the specificity of SER for Mϕ, rosetting was also carried out on cells in suspension. In three independent experiments with collagenase-digested bone marrow, 1–3% of nucleated cells formed rosettes, 100% of which were RBMM as assessed by size, morphology, and immunoperoxidase labeling with F4/80 (not shown). The proportion of RBMM that formed rosettes in suspension was 80–90%, values that were simliar to those seen with adherent RBMM. These results therefore suggested that SER is Mϕ restricted.

Characteristics of the Interaction between RBMM and E. To investigate the nature of the surface molecules on RBMM and E that mediate rosetting we tested various conditions and reagents for their ability to affect binding. These included temperature and cation dependence, effects of metabolic inhibitors,

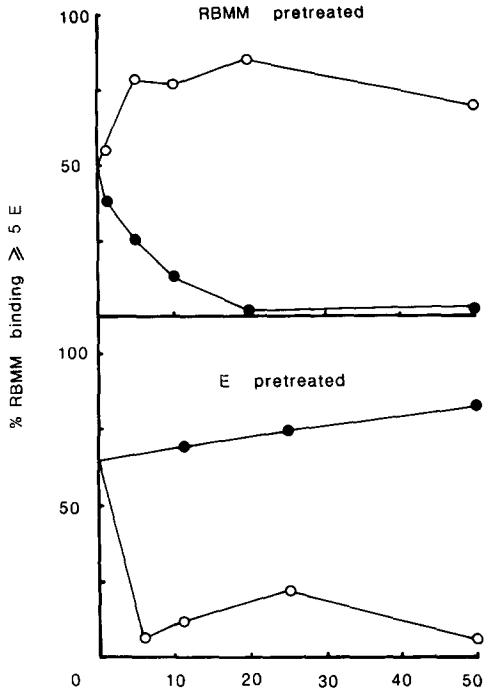

FIGURE 2. Differential effect of trypsin (*filled circle*) or neuraminidase (*open circle*) pretreatment of RBMM (*top*) or E (*bottom*) on rosette formation. The data show mean values of duplicate coverslips. Individual counts were <10% of the mean and are omitted for clarity. Simliar results were obtained in three independent experiments.

and pretreatment of RBMM or E with proteolytic enzymes or *V. cholerae* neuraminidase (Fig. 2). Rosetting was unaffected over the temperature range 0–37°C (0, 4, 22, and 37°C) and was independent of divalent cations, since addition of 0–10 mM EDTA to RPMI did not influence binding. Binding was also unaltered in calcium and magnesium-free PBS containing 1 mM EDTA, although RBMM became rounded under these conditions (not shown). Finally, rosetting was unaffected in the presence of the metabolic inhibitors sodium azide and iodoacetate up to 10 mM.

SER was found to be labile to *S. aureus* V8 protease and trypsin (Fig. 2, *top*), but resistant to the collagenase and DNase used in isolation of RBMM. The dose-dependent inhibition observed with trypsin was not due to a toxic effect on RBMM since rosetting to antibody-coated E was unimpaired, presumably via the trypsin-resistant Fc receptor (12, not shown). In contrast to protease and trypsin, treatment of RBMM with neuraminidase resulted in enhancement of rosetting (Fig. 2, *top*). When E were pretreated with trypsin or neuraminidase, we saw precisely the opposite pattern (Fig. 2, *bottom*). Thus, trypsinization led to a dose-dependent enhancement of binding, whereas neuraminidase treatment inhibited binding by up to 90% at the lowest concentration tested, 6 U/ml. Similar results were obtained with *V. cholerae* neuraminidase supplied by Calbiochem-Behring, which inhibited >90% at an equivalent low concentration (<0.01 Behringwerke

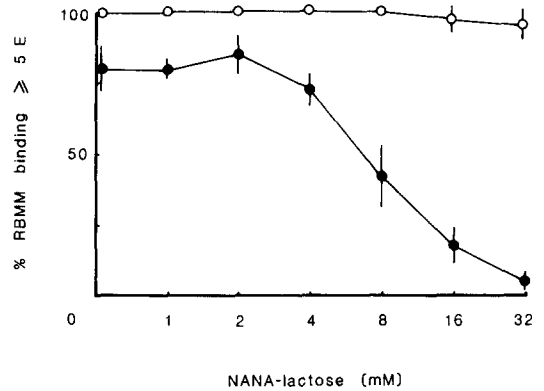

FIGURE 3. Inhibition by NANA-lactose of E (*filled circles*) but not EIgG (*open circles*) binding to RBMM. Data show means ± 1 SD of triplicate coverslips. Similar results were obtained in two independent experiments. No inhibition was seen with the following mono- and disaccharides, all tested at 100 mM; D-glucose, D-galactose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, D-fucose, D-mannose, *N*-acetyl neuraminic acid, α-lactose, and β-lactose.

U/ml). Sialylated components on the surface of E therefore appeared to be required for the interaction with SER on RBMM.

Lectin-like Properties of SER. To investigate the possible lectin-like nature of SER, we first determined whether various sugars could inhibit rosetting (for details see legend to Fig. 3). Of the mono- and disaccharides studied, none had any effect on rosetting at concentrations up to 100 mM. However, the trisaccharide NANA-lactose, purified either from human or bovine colostrum, gave 50% inhibition at 5–10 mM (Figs. 1*C* and 3). 35 mM was required for 100% inhibition. This was not due to nonspecific toxic effects, since RBMM treated with NANA-lactose were morphologically intact (Fig. 1*C*) and rosette formation with EIgG was virtually unaffected (Fig. 1*D* and Fig. 3).

In view of the seeming importance of sialylated components on E, we also tested the inhibitory effect of the mono- and disialylated gangliosides GM1 and GD1a, respectively, and of the richly sialylated glycoproteins fetuin, orosomucoid and ovine submaxillary mucin. GM1 gave no inhibition at the highest concentration tested (1,000 μg/ml, 650 μM) but GD1a inhibited binding by 50% at 20 μg/ml (11 μM) and by 100% at ~100 μg/ml. Inhibition was not accompanied by toxic effects and RBMM remained fully viable throughout. A 30-min preincubation of RBMM with GD1a, followed by washout, resulted in inhibition of rosetting at similar concentrations. This suggests that GD1a mediates its effects by interacting with RBMM. Of the sialoglycoproteins tested, fetuin or orosomucoid at concentrations up to 1,000 μg/ml (20 μM) had no effect on rosetting, but ovine submaxillary mucin gave 50% inhibition at 500 μg/ml (3 μM). This was not thought to be significant since higher concentrations were toxic.

Previously characterised lectin-like receptors on Mϕ include the mannosyl-fucosyl receptor (MFR) and galactose receptor (13, 14). To exclude a role for either of these in rosette formation, we tested the effect of specific neoglycoconjugate inhibitors. We saw no inhibition with either mannosylated or galactosylated BSA at 100 μg/ml (1.5 μM), concentrations that completely inhibit MFR and the galactose receptor, respectively (15, 16). In addition, pre- or coincubation

TABLE I
Inverse Expression of SER and Ia Antigens on RBMM

Exp.	Strain	n‡	Ia negative RBMM*			Weak Ia+ RBMM			Strong Ia+ RBMM		
			Total RBMM	≥5 E	E per RBMM	Total RBMM	≥5 E	E per RBMM	Total RBMM	≥5 E	E per RBMM
			%	%		%	%		%	%	
1	C57BL/6§	234	79	96	47 ± 29‖	13	72	9 ± 17	8	32	2 ± 3
2	C57BL/6	215	72	98	50 ± 41	16	81	25 ± 22	12	12	2 ± 3
3	C57BL/6	138	46	97	50 ± 33	14	33	16 ± 16	40	33	4 ± 5
4	BALB/C¶	248	36	99	43 ± 27	34	77	23 ± 21	30	3	1 ± 2

* Expression of Ia antigens on individual RBMM, determined by immunocytochemistry using mAb M5/114 (anti-I-$A^{b,d,q}$, I-$E^{d,k}$).
‡ Total number of RBMM counted on duplicate coverslips.
§ I-A^b haplotype.
‖ Mean ± 1 SD derived from counts of E on individual RBMM.
¶ I-A^d/I-E^d haplotype.

with 100% homologous plasma or serum had no detectable effect on binding of E. Finally, saturating concentrations of the mAbs F4/80, M5/114 (Ia antigen), 2.4G2 (FcR II), or M1/70 (CR3) did not affect rosetting.

Inverse Expression of SER and Ia Antigens on RBMM. The heterogeneity in expression of SER on RBMM was a noticeable feature in all experiments. As heterogeneity of a similar kind had previously been seen (5) for expression of Ia antigens on RBMM, we investigated the relationship between these two markers by employing a double-labeling procedure of rosetting followed by immunoperoxidase staining with mAb M5/114 to detect Ia antigens. Control experiments showed that prior rosetting with E did not alter the proportion of RBMM-expressing Ia antigens, as assessed by immunocytochemistry (not shown). In four independent experiments the proportion of RBMM expressing Ia antigens varied from 21–64%. In each experiment there was a clear inverse correlation between expression of SER and Ia antigens on RBMM isolated from either C57BL/6 (H-2^b) or BALB/c (H-2^d) mouse strains (Table I). This was most clear if the Ia^- population was compared with the strongly Ia^+ RBMM. Thus, in all experiments, 96–100% of Ia^- RBMM bound ≥5 E while only 3–33% of the strongly Ia^+ population formed rosettes. The weakly Ia^+ population showed variable E rosetting, although the mean values of E bound per RBMM were intermediate between the Ia^- and strongly Ia^+ RBMM subpopulations (Table I).

SER Is Expressed Transiently on RBMM. During attempts to show biosynthesis of SER by RBMM it was found that reexpression after trypsinization did not exceed 10% of initial levels over a 48-h period of cultivation in RPMI plus 10% FCS (not shown). However, examination of SER expression on RBMM not treated with trypsin also showed that binding activity was gradually lost over a 3-d period with a half-life of ~24 h (Fig. 4). The monocytes that initially contaminated the RBMM preparations survived poorly and were not included in the analysis. The loss of SER was accompanied by other phenotypic changes, which included increased expression of Mac-1 antigen, initially undetectable, and a transient increase in expression of Ia antigen. There was no change in expression of F4/80 antigen, which remained on 100% of RBMM throughout culture (Fig. 4). RBMM also exhibited morphological changes during cultivation, with retraction of the extensive plasma membrane processes (not shown). This was

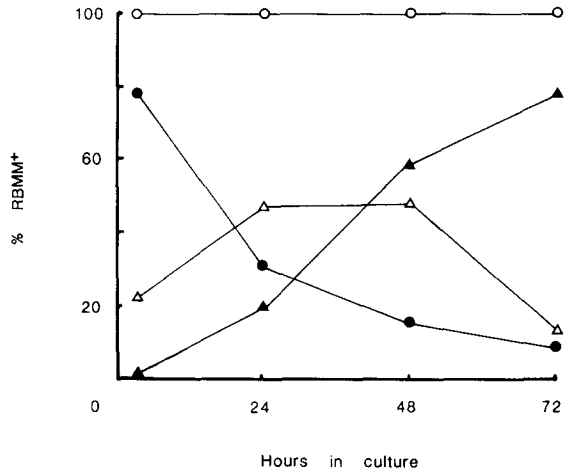

FIGURE 4. Decline in expression of SER (*filled circles*) on RBMM cultivated in RPMI plus 10% FCS compared with expression of Ia (*open triangles*), Mac-1 (*filled triangles*), and F4/80 (*open circles*) antigens. The data show mean percentage of RBMM$^+$ for each marker (percent binding ≥5 E or percent with clearly visible diaminobenzidine reaction product). 200 RBMM were counted on each of duplicate coverslips; contaminating monocytes were not included. Individual counts were within 10% of mean and are omitted for clarity. Similar results were obtained in three independent experiments.

accompanied by gradual detachment of nonviable RBMM with ~50% remaining at 72 h. Precisely the same pattern of phenotypic changes was seen in three independent experiments. In conclusion, SER levels declined in culture with a half-life of ~24 h. This could not be accounted for by a population shift and was accompanied by changes in expression of Mac-1 and Ia antigens, but not F4/80.

Distribution of SER on Monocytes and Various Mϕ Populations. The properties and differential expression of SER on RBMM compared with RPM suggested that it could be involved in adhesive cellular interactions within bone marrow. It was therefore important to determine the distribution of SER on other deeply embedded stromal tissue Mϕ populations and compare this with expression on other "free" Mϕ obtained by lavage from serous cavities and bronchoalveolar spaces (Table II). To distinguish Mϕ from other adherent cells, preparations were rosetted and then stained immunocytochemically with the Mϕ-specific mAb, F4/80. Control experiments showed that immunocytochemical detection of F4/80 was unaffected by prior rosetting (not shown). This double-labeling procedure was essential with stromal tissue Mϕ isolated by collagenase digestion from liver, spleen, mesenteric lymph nodes, and thymus, which were often contaminated by a variety of other cell types (e. g., endothelial cells, fibroblasts, dendritic cells, and epithelial cells).

~50% of Kupffer cells were SER$^+$, but rosetting was of lower intensity than that seen with RBMM. Contaminating F4/80$^-$ endothelial cells did not bind E. With spleen, 25–55% of the F4/80$^+$ adherent cells formed rosettes, with similar intensity to that of Kupffer cells. This was seen predominantly on the large, well-spread population and was normally absent from the smaller immature Mϕ and monocytes. Occasionally, well-spread F4/80$^-$ cells with Mϕ morphology formed rosettes, but F4/80$^-$ cells with the morphology of Steinman-Cohn spleen den-

TABLE II

Expression of SER on Different Mϕ Populations and Monocytes after Adherence to Glass

Tissue	Number of experiments	Adherent cells F4/80+*	F4/80+ cells binding ≥5 E*‡
		%	%
Bone marrow	4	60–81§	64 ± 11‖
Liver	4	50–90	51 ± 9
Mesenteric lymph node	3	53–60	55 ± 11
Spleen	4	28–65	29 ± 9
Thymus	2	55–65	3 ± 1
Peritoneal cavity			
Resident	9	90–100	7 ± 6
Thioglycollate	10	95–100	3 ± 4
BCG-activated	2	95–100	6 ± 2
C. parvum–activated	2	95–100	5 ± 2
Pleural cavity	3	90–100	0 ± 0
Bronchoalveolar spaces	3	95–100	0 ± 0
Blood	5	80–90	0 ± 0

* Based on counts of duplicate coverslips in each experiment and includes small F4/80+ Mϕ and monocyte-like cells. 100–200 F4/80+ cells counted per coverslip.

‡ Positive rosetting of E to F4/80− cells was seen only in spleen where it constituted ~5% of total rosettes.

§ Range of values from the indicated number of experiments.

‖ Mean ± 1 SD of individual coverslips (two per experiment).

dritic cells (17) did not bind E (not shown). With collagenase-digested mesenteric lymph nodes, rosetting was again seen predominantly on the large, well-spread stromal Mϕ and was of similar intensity to that of RBMM. With adult thymus, the majority of F4/80+ adherent cells were small cells resembling immature Mϕ and expression of SER was restricted to the few stromal-type Mϕ. With the peritoneal cavity, a variable, but usually low degree of rosetting (<10%), was seen with either resident, inflammatory, or activated Mϕ. Pleural Mϕ, bronchoalveolar Mϕ, and blood monocytes were all SER−. Finally, in two experiments with spleen, rosetting was also carried out in suspension. 5–6% of nucleated spleen cells bound ≥5 E, and 90% of the rosetting cells were F4/80+. The F4/80−, SER+ subpopulation consisted of large phagocytic cells with oval nuclei and a nuclear/cytoplasmic ratio of ≪1. They may correspond to F4/80− marginal zone Mϕ (18). Cells with lymphocyte morphology did not bind E.

In conclusion, SER was expressed differentially on other stromal tissue Mϕ populations, in addition to RBMM, and appeared to be Mϕ specific. Of the tissues examined, we saw the most intense rosetting on RBMM and mesenteric lymph node Mϕ; intermediate levels with liver Mϕ and stromal splenic Mϕ; but we saw low levels of SER on monocytes and Mϕ isolated from the thymus, serous cavities, and alveolar spaces.

Discussion

In this study we have characterized a novel lectin-like, Mϕ-restricted receptor, with specificity for sialylated ligand(s) on sheep E. This hemagglutinin mediates

binding, but not ingestion, of E and is differentially expressed, being present at high levels on a majority of murine RBMM and stromal lymph node macrophages, at intermediate levels on liver and stromal splenic Mϕ, but at low or undetectable levels on blood monocytes and thymic, peritoneal, pleural, and bronchoalveolar Mϕ. We propose that this hemagglutinin is involved in nonphagocytic cellular interactions within tissues.

E are commonly used as carriers of Ig or complement fragments to detect FcR and complement receptors, respectively, on myeloid and lymphoid cells. It is therefore, perhaps, surprising that background rosetting with unopsonized control E has not been reported in previous studies of murine tissue Mϕ. However, effective isolation of Mϕ from solid tissues requires the use of tissue-dispersing enzymes so that methods based on mechanical disruption would select for the nonstellate, SER^- Mϕ. Even in those studies that use dispersing enzymes, nonspecific proteases, either in the enzyme preparation or released from tissues, could remove the receptor. In addition, since bound E are not ingested via SER, they could be easily removed during pipetting procedures, which were avoided in our assays.

In contrast to the mouse, the formation of rosettes with unopsonized E is a well-recognized phenomenon with human T lymphocytes (19, 20). By several criteria, the ligand on E recognized by human T cells appears to be distinct from that recognized by murine macrophages. These include the converse effects of neuraminidase and trypsin pretreatment of E as well as the temperature and metabolic requirements for rosette formation with human T cells (21). The precise nature of the ligand on E that interacts with murine macrophages is unclear, although the extreme sensitivity to neuraminidase provides evidence that sialic acid is a minimal requirement. It is possible that the recognized sialic acid is associated with a ganglioside rather than a glycopeptide, since there was potent inhibition by the ganglioside, GD1a, but not by a variety of heavily sialylated glycoproteins. The inhibition mediated by GD1a was unlikely to be a nonspecific detergent effect since high concentrations of the related ganglioside, GM1, gave no inhibition. We have not carried out an extensive survey of different gangliosides because the mechanism of inhibition is likely to be complex and for meaningful comparisons it is necessary to use ceramide-free oligosaccharides. It is probable that the inhibition mediated by millimolar concentrations of the trisaccharide, NANA-lactose, reflects a low-affinity interaction with SER. This could not be simply a charge effect, because *N*-acetyl neuraminic acid failed to inhibit binding at similar concentrations, thereby implying that either the correct anomeric structure of sialic acid (22) or adjoining galactose and/or glucose residues are required. Further studies are clearly needed to define the structural requirements for optimal inhibition of SER.

SER appears to be unrelated to the previously well-characterized Mϕ lectin-like receptors, MFR, and galactose receptor (13, 14), but it may be related to agglutinins described on rat bronchoalveolar and peritoneal Mϕ and on the murine Mϕ-like cell line, Mml, which recognize sialylated components on ganglioside-treated E and quail erythrocytes, respectively (23, 24). Although the properties of both receptors are similar to SER, the differential expression on tissue Mϕ populations was not reported previously.

The lability of SER to trypsin suggests it is a protein, but because of its transient expression in culture, we could not determine whether RBMM synthesize or acquire the hemagglutinin. In recent experiments (our unpublished observations), however, we have found that SER can be induced readily by cultivation in the presence of mouse serum, rather than in FCS as used here, and that reexpression after trypsinisation is sensitive to cycloheximide, suggesting that RBMM synthesize SER. In the present study, the loss of SER was accompanied by changes in expression of Mac-1 and Ia antigens that probably reflect rapid in vitro dedifferentiation. At the beginning of culture, however, there was a striking inverse correlation between expression of SER and Ia antigens that points to differential in vivo regulation, the significance of which is unknown.

In conclusion, the high levels of SER on deeply embedded stromal Mϕ and the absence of ingestion suggest that the hemagglutinin may be involved in nonphagocytic interactions within tissues. In bone marrow, for example, SER on RBMM could interact with the attached hematopoietic cells to influence their growth and differentiation, a possibility that has recently been strengthened by our finding that bone marrow cells express an appropriate sialylated ligand (our unpublished observations). The heterogeneity in SER levels detected among various tissues may reflect the differing proportions of immature macrophages as well as the effects of local and systemic modulators that regulate SER expression. Interestingly, the Mϕ populations that exhibited lowest levels of SER were obtained by lavage from serous cavities or bronchoalveolar spaces. This implies that the majority of SER-bearing Mϕ are "fixed" within tissues, either through adhesion to extracellular matrix components or by cellular interactions. Further studies are needed to establish whether SER is expressed after fixation or whether it contributes directly to this process.

Summary

We describe a novel hemagglutinin which is differentially expressed on murine stromal tissue macrophages. Resident bone marrow macrophages (RBMM), which are physically associated with immature, proliferating hematopoietic cells in vivo, formed striking rosettes with unopsonized sheep erythrocytes (E) in vitro, unlike resident peritoneal macrophages (RPM). Binding of E was macrophage (Mϕ) specific, not accompanied by ingestion and independent of temperature (0–37°C), divalent cations, and the metabolic inhibitors azide and iodoacetate. Pretreatment of RBMM with trypsin prevented rosette formation, but neuraminidase enhanced it. Conversely, binding was virtually abrogated if E were pretreated with neuraminidase, whereas trypsin pretreatment of the ligand resulted in a slight enhancement. The lectin-like nature of the E receptor (SER), with specificity for sialylated glycoconjugates, was consistent with the inhibition of binding we saw with neuraminyllactose or the ganglioside GD1a (50% inhibition at 5–10 mM and 11 μM, respectively).

Expression of SER on freshly isolated RBMM was heterogeneous and exhibited a striking inverse correlation with expression of Ia antigens. During cultivation in 10% FCS, levels of SER on RBMM declined with a half-life of ~24 h. Other cell surface changes induced by cultivation included a transient increase in expression of Ia antigen and acquisition of Mac-1. To determine whether SER

was expressed on other stromal Mϕ populations, adherent cells were isolated from various tissues by collagenase digestion or lavage. Binding of E was highest on RBMM and lymph node stromal Mϕ, at intermediate levels on Kupffer cells and splenic stromal Mϕ, but was low or undetectable on blood monocytes and thymic, peritoneal, pleural, and bronchoalveolar Mϕ. SER therefore appeared to be expressed on certain Mϕ populations embedded in solid tissues but was largely absent from Mϕ recoverable by lavage. Its absence from monocytes implies that SER is acquired by Mϕ after entering tissues where it may perform adhesive functions. In bone marrow, SER on RBMM could interact with an appropriate sialylated ligand on murine hematopoietic cells, and could influence their rate of growth and differentiation.

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