# MOUSE MACROPHAGE HEMAGGLUTININ (SHEEP ERYTHROCYTE RECEPTOR) WITH SPECIFICITY FOR SIALYLATED GLYCOCONJUGATES CHARACTERIZED BY A MONOCLONAL ANTIBODY

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The functions of resident macrophages (M∅)[1] in hematopoietic and lymphoid organs are poorly understood. In several species, in situ immunocytochemical staining with M∅-specific mAbs has demonstrated that these tissues contain large numbers of stromal M∅, even in the absence of overt inflammation or immune stimulation (1–6). We and others have previously speculated that stromal M∅ may perform diverse local homeostatic functions, including regulation of growth, differentiation and movement of hematopoietic and lymphoid cells (7, 8). Our experimental approach has been to isolate the relevant M∅ populations and determine whether they possess novel features that could be involved in cellular interactions.

We have focused on murine bone marrow, since this tissue contains an arborizing network of stromal M∅ that establish intimate adhesive contacts with proliferating erythroid and myeloid cells (1, 7). After their isolation, the majority of resident bone marrow M∅ express a novel receptor, sheep erythrocyte receptor (SER), which mediates the binding, but not ingestion, of unopsonized sheep erythrocytes (E) (9). Since it is possible that SER normally interacts with a homologous ligand on mouse bone marrow cells it was of interest to characterize its properties and distribution.

SER is a divalent cation-independent, lectin-like hemagglutinin that recognizes sialylated glycoconjugates on the erythrocyte surface. Its properties are similar to the "ganglioside receptor" on rat M∅ described by Riedl and colleagues (10). In addition to resident bone marrow M∅, the receptor is expressed at high levels on isolated stromal lymph node M∅ but it is low or undetectable on monocytes or peritoneal, pleural, and bronchoalveolar M∅. These features indicate that SER may be involved in interactions of stromal M∅ with ligands on neighboring cells or extracellular matrix components.

[1] *Abbreviations used in this paper:* Ag, antigen; CR3, complement receptor type 3; DAB, diaminobenzidine; LC, leukocyte common; M∅, macrophage; SER, sheep erythrocyte receptor; SER-4 Ag, the antigen recognized by mAb SER-4; TPM, thioglycollate-elicited peritoneal macrophages.

To understand the nature and functions of SER, it would clearly be advantageous to raise an mAb to the receptor. The recent observation that high levels of SER are induced on peritoneal M∅ cultivated in mouse serum provided us with a suitable source of antigenic material and a rapid method for screening inhibitory mAbs (11). In this paper we describe the isolation of mAb SER-4, characterize the molecular nature of SER, and determine its distribution on M∅ in various tissues.

## Materials and Methods

*Animals.* Female C57Bl/6 mice were bred at the Sir William Dunn School of Pathology, Oxford University and used at 2–4 mo. Specific pathogen–free female AO rats were bred in the MRC Cellular Immunology Unit, Oxford University and used at 3–6 mo.

*Reagents.* M1/9.3, a rat pan antileukocyte common (LC) antigen (Ag) mAb (12), was provided by Dr. R. S. Steinman, The Rockefeller University (New York, NY), MOMA-1, a rat mAb that recognizes marginal metallophils of the spleen (13), was kindly supplied by Dr. G. Kraal, Free University, Amsterdam, The Netherlands. 1A10, a rat mAb directed to an Ag on guinea pig neutrophils (14) from Dr. H. Rosen in our laboratory was used as a negative control in immunoprecipitation and immunocytochemistry experiments. From Sigma Chemical Co. (Poole, United Kingdom) we obtained BSA, glucose oxidase, iodoacetamide, PMSF, EDTA, pepstatin, and soybean trypsin inhibitor. Lactoperoxidase was obtained from Boehringer Corp., Lewes, United Kingdom. FCS was purchased from Imperial Laboratories, Salisbury, United Kingdom. Iscove's modification of Dulbecco's Medium and Hepes buffer were obtained from Gibco Laboratories, Paisley, Scotland. Carrier-free Na$^{125}$I, [$^{35}$S]methionine (code SJ.204) and [$^{35}$S]cysteine (code SJ.232) were purchased from Amersham International PLC, Amersham, UK. Protein A-Sepharose was from Pharmacia Fine Chemicals, Hounslow, UK.

*Cells.* Thioglycollate-elicited peritoneal M∅ (TPM) were obtained 4 d after intraperitoneal injection of 1 ml Brewer's complete thioglycollate broth. Resident bone marrow M∅ were isolated by collagenase digestion of femoral bone marrow plugs, selective adherence of purified clusters to glass coverslips, and removal of attached hematopoietic cells (7).

*Sheep Erythrocyte Rosetting Assays.* These were carried out as described previously (11). Briefly, 2 × 10$^4$ TPM were added to each well of a 96-well tissue culture microtiter plate (Falcon Labware, Lincoln Park, NJ) and cultured for 3–4 d in 100 μl DME containing 15% mouse serum prepared as previously described (11). After washing with DME + 0.1% BSA, 20 mM Hepes, sheep E were added at 0.5% vol/vol and the plates incubated at 37°C for 30 min. Nonadherent sheep E were removed by repeated washing with medium and the percentage of M∅ binding >4 E was determined. In some experiments, rosetting assays were carried out with M∅ on coverslips (9).

*Production of mAb SER-4.* AO rats were immunized intramuscularly three times at 10-d intervals with 10$^7$ TPM that had been cultured for 3 d in 15% mouse serum to induce SER (11). Before injection, TPM were scraped from dishes and washed three times in PBS to remove mouse serum. The first injection was with complete and subsequent injections with IFA. Rats were boosted intravenously with 5 × 10$^6$ mouse serum-induced TPM 3 d before fusion of spleen cells with the rat myeloma line Y3 (15). Hybridoma supernatants were screened for their ability to prevent rosette formation to mouse serum–induced TPM using the microtiter assay described above. One positive supernatant was identified from a total of >2,500 in four separate fusions and the hybridoma, designated SER-4, was cloned three times by limiting dilution. The isotype of mAb SER-4 was determined by Ouchterlony gel diffusion analysis using antisera directed against rat IgG subclasses, kindly supplied by Dr. H. Bazin (Catholic University, Louvain, Belgium).

*Purification of IgG and Its Fragments from SER-4.* SER-4 was grown as an ascites in pristane-primed AO rats, and the IgG was purified by sodium sulphate precipitation and anion-exchange chromatography using DEAE-Sephacel (Pharmacia Fine Chemicals). F(ab')$_2$ and Fab fragments were prepared as described by Rousseaux et al. for rat IgG2a (16). Purity of IgG, F(ab')$_2$ and Fab was >95% when assessed by SDS-PAGE and Coomassie Brilliant Blue

staining, using 10% acrylamide gels under reducing and nonreducing conditions. Protein concentrations were determined by measuring absorbance at 280 nM, assuming an extinction coefficient of 1.4.

*Comparison of SER Expression with SER-4 Ag.* TPM were plated at $10^5$/well in 96-well tissue culture microtiter plates in the defined serum-free medium, HB102 (New England Nuclear, Boston, MA) and allowed to adhere for 90 min. This and subsequent incubations were at 37°C and 5% $CO_2$ in a fully humidified incubator. Supernatants were removed and replaced with HB102 medium containing mouse serum at 20, 5, or 0%. SER assays were carried out in quadruplicate on days 0, 1, 2, and 4. Rosettes were fixed in 0.125% glutaraldehyde for 10 min and permeabilized in methanol for 30 min. The relative numbers of sheep E bound/well were determined by measuring the pseudoperoxidase activity of hemoglobin (17), as follows. PBS containing 10 mM imidazole, 0.5 mg/ml diaminobenzidine (DAB), and 0.02% $H_2O_2$ (pH 7.4), was added to wells. After 1 h at room temperature absorbance of the reaction product was measured at 410 nm on a Titertek plate reader (Dynatech Laboratories, Inc., Alexandria, VA).

To determine the number of cells in each well, the nuclei of methanol-fixed TPM in separate wells were stained for 1 min with 1% crystal violet in 70% methanol, followed by extensive rinsing with tap water. The absorbance at 570 nm was determined on the Titertek plate reader and converted to cell number using a linear standard curve, obtained by plating serial dilutions of TPM. The relative number of sheep E bound per M∅ was calculated and expressed as an E/M∅ index = OD (DAB)/OD (Crystal violet).

The remaining wells on each row were used for determination of SER-4 Ag site number. Wells were rinsed with FCS, air dried, and the plates were stored desiccated at −20°C. Immediately before assay, TPM were fixed for 10 min in methanol and nonspecific binding sites blocked by incubation in 10% rat serum for 30 min. Wells were incubated for 60 min at 4°C with 50 µl of a predetermined saturating concentration (10 µg/ml) of $^{125}$I-SER-4 Fab, specific activity 1.3 µCi/µg, labeled by the chloramine T method (18). Specific binding was determined by subtracting counts from separate wells that had been preincubated with 30 µg/ml unlabeled SER-4 IgG before addition of $^{125}$I-SER-4 Fab. As an internal control for site number analysis, binding assays were also carried out with $^{125}$I-Fab fragments of the anti-CR3 mAb, 5C6, provided by Dr. H. Rosen (19). Bound radioactivity was solubilized in 0.5% NaOH and counted in a Packard 5210 gamma counter.

*Immunocytochemistry.* In preliminary experiments the antigenic determinant recognized by mAb SER-4 was destroyed by the fixatives glutaraldehyde and paraformaldehyde but not ethanol, methanol, or acetone. 7-µM cryostat sections were therefore fixed for 10 min in acetone at room temperature and stained with a 1:5 dilution of hybridoma tissue culture supernatant using an avidin-biotin-peroxidase detection kit (Vectastain; Vector Laboratories, Peterborough, UK). Negative controls included omission of first antibody and use of rat mAbs directed to unrelated Ag.

*Immunoprecipitation.* $10^7$ TPM were cultured for 2 d in 10-cm diameter tissue culture petri dishes at a concentration of $10^6$/ml in DME + 10% mouse serum. For metabolic labeling, the medium was changed to methionine- and cysteine-free Eagle's medium with 10% mouse serum that had been desalted into the same medium using PD-10 columns (Pharmacia Fine Chemicals). [$^{35}$S]Methionine and [$^{35}$S]cysteine were each added at 25 µCi/ml, final concentration, and the cells were incubated for a further 16 h. For surface labeling, TPM, adherent to 10-cm diameter dishes, were iodinated by the lactoperoxidase-glucose oxidase method for 30 min at 4°C (20). After either method of labeling, TPM were washed extensively and solubilized in 10 mM Tris lysis buffer, pH 8.0, containing 150 mM NaCl, 2% NP-40, 2 mM PMSF, 5 mM EDTA, 5 mM iodoacetamide, 100 µg/ml soybean trypsin inhibitor, 1 µg/ml pepstatin, and 0.5% BSA. Nuclei and other insoluble materials were removed by centrifugation at 10,000 g and immunoprecipitations carried out as described (19). The samples were electrophoresed on 6.5% polyacrylamide gels according to Laemmli (21). $^{14}$C-methylated protein molecular weight markers (code CFA 626) were obtained from Amersham International PLC.

*Western Blots.* TPM were cultured for 3 d and lysates were prepared as described above. Spleen, mesenteric lymph nodes, thymus, and femoral bone marrow plugs were obtained

from a single mouse and added to 1 ml of 10 mM Tris, pH 8.0, containing 150 mM NaCl
and protease inhibitors. The tissues were disrupted using an electrically driven homogenizer
(Polytron; Kinematica GmbH, Lucerne, Switzerland) and solubilized by addition of an equal
volume of nonreducing 2× SDS-PAGE sample buffer. Reduction with $\beta$-mercaptoethanol
resulted in loss of antigenic activity. After removal of insoluble material by centrifugation
at 10,000 g, 35 $\mu$l of the lysates were electrophoresed on 6.5% polyacrylamide gels. Proteins
were transferred to nitrocellulose as described (22) and probed with 0.5 $\mu$g/ml ^{125}I-SER-4
IgG, prepared by the chloramine T method (18). Specificity was determined by comparing
the signals obtained in the presence or absence of 100-fold excess of unlabeled SER-4 IgG.

## Results

*Isolation of an mAb Directed to SER.*    To obtain an mAb to SER, our strategy was
to immunize rats with mouse serum–induced TPM expressing high levels of SER,
as determined by sheep E binding, and then to screen hybridoma supernatants for
their ability to block sheep E binding to induced TPM (11). From a total of four
fusions, one positive hybridoma was identified and designated SER-4. Tissue cul-
ture supernatant from SER-4 was able to completely inhibit binding of sheep E to
mouse serum–induced TPM (Fig. 1). Complete inhibition of sheep E binding was
also observed with resident bone marrow M$\emptyset$ on which the hemagglutinin was first
described (not shown). Inhibition of SER by SER-4 supernatant was not accompa-
nied by visible signs of toxicity or changes in morphology.

SER-4 was cloned three times by limiting dilution and was found to secrete an
mAb of the rat IgG2a subclass by Ouchterlony analysis. After purification, the in-
tact immunoglobulin was able to inhibit sheep E binding completely at ~1 $\mu$g/ml
(Fig. 2). In comparison, F(ab')$_2$ fragments of SER-4 mAb gave a maximum of only
50–60% inhibition, while Fab fragments had no effect on rosetting, even at concen-
trations up to 100 $\mu$g/ml (Fig. 2). The failure of Fab to inhibit binding of sheep E
was not due to its lower avidity, since at 40 $\mu$g/ml it completely inhibited binding
of a saturating concontration (1 $\mu$g/ml) of SER-4 IgG to TPM. In addition, [125]I-
SER-4 Fab bound to induced TPM in a specific and saturable manner, with max-
imal binding at 10 $\mu$g/ml (not shown).

*Comparison of Expression of SER with the SER-4 Ag.*    Our previous studies have demon-
strated that the in vitro expression of SER on TPM can be regulated reversibly by
mouse serum in a dose-dependent fashion (11). It was therefore of interest to com-
pare the binding of sheep E with expression of the SER-4 Ag by TPM cultured in
a range of mouse serum concentrations. Antigenic site number analysis was carried
out by direct binding assay with ^{125}I-SER-4 Fab on methanol-fixed TPM. This
measures the total amount of SER-4 Ag, both intra- and extracellular. It can be seen
in Fig. 3 that expression of SER and the SER-4 Ag were closely correlated throughout
the course of the experiment. At the outset of culture, TPM bound an average of
4 × 10⁴ SER-4 Fab molecules/cell and there was negligible binding of sheep E. The
highest levels of both SER-4 Ag and SER expression were observed on day 2 of cul-
ture in 20% mouse serum. At this time TPM bound an average of ~10⁶ SER-4 Fab
molecules/cell, representing a 25-fold increase in antigenic site number. In compar-
ison, levels of SER activity (sheep E binding) increased ~10-fold. As an internal
control for the site number analysis, binding assays were also carried out with Fab
fragments of 5C6 anti CR3 mAb (19). On day 2, TPM bound on average of 2 ×
10⁵ molecules of [125]I-5C6-Fab, irrespective of the concentration of mouse serum used
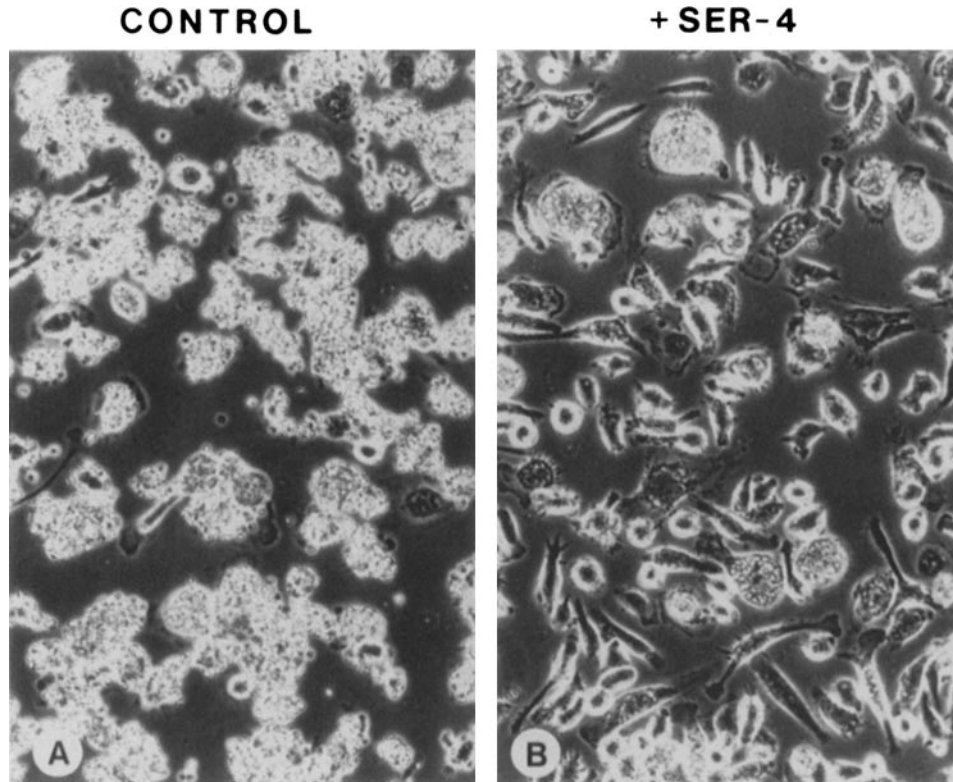
**CONTROL**                    **+ SER-4**



FIGURE 1.   Inhibition of SER by mAb SER-4. Phase contrast micrographs of rosetting assays with mouse serum-induced TPM in the absence (A) or presence (B) of SER-4 mAb tissue culture supernatant. TPM were cultured on glass coverslips for 3 d in 15% mouse serum to induce SER. They were then incubated for 1 h in the presence or absence of SER-4 mAb tissue culture supernatant. After extensive washing, sheep E were added at 0.5% vol/vol. After 30 min at 37°C unbound sheep E were removed and the preparations were fixed in 1% glutaraldehyde before examination. (A) The majority of TPM rosette intensely with sheep E. (B) Erythrocyte binding is completely inhibited after preincubation with SER-4 mAb tissue culture supernatant.
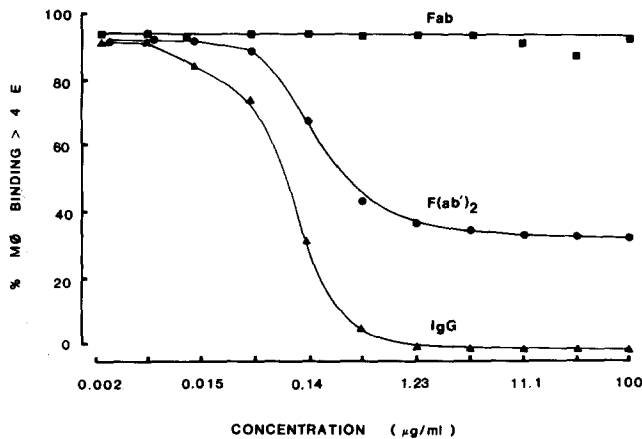


FIGURE 2.   Inhibition of SER by mAb SER-4 IgG, F(ab')₂, and Fab fragments. TPM were induced to express SER by culture in 15% mouse serum for 3 d on microtiter plates. Wells were incubated for 1 h with threefold serial dilutions of IgG, F(ab')₂, or Fab, washed extensively, and assayed for sheep E binding. The data show mean values of duplicate wells. Similar results were obtained in three independent experiments.
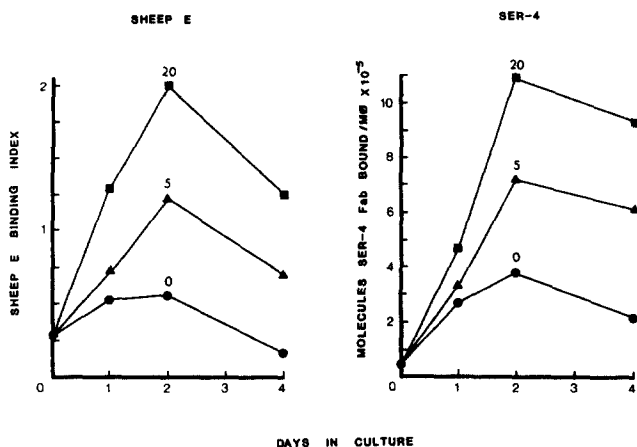
Figure 3. Comparison of SER expression with SER-4 Ag in cultured TPM. TPM were cultured for up to 4 d on microtiter plates in the presence of 20, 5, or 0% mouse serum. The level of SER (left) is expressed as the sheep E binding index. This is a measure of the average number of E bound per M$\emptyset$, determined by optical density. For E, the pseudoperoxidase activity of hemoglobin was measured at 410 nm, using DAB as substrate, and the number of M$\emptyset$ per well was measured at 570 nm after nuclear staining with crystal violet. The sheep E binding index was calculated as $OD_{410}/OD_{570}$. The level of SER-4 Ag (right) was determined by direct radioimmune binding assay, using a saturating concentration of $^{125}I$-SER-4 Fab. Measurements of SER and SER-4 Ag were carried out at the beginning of culture and on days 1, 2, and 4. Serum was removed from all remaining wells on day 2 in order to reverse the expression of SER.

for culture. This is in agreement with previous estimates of CR3 site number on M$\emptyset$ (23). After removal of serum after 48 h, the levels of SER-4 Ag and SER declined in parallel. These results support the notion that the Ag recognized by SER-4 mAb is either closely related to, or identical with, SER.

*Characterization of the Antigen Recognized by mAb SER-4.*    After metabolic labeling of mouse serum-induced TPM with a mixture of [$^{35}S$]methionine and [$^{35}S$]cysteine, mAb SER-4 was found to immunoprecipitate two species of Ag determined by SDS-PAGE not seen with control mAb 1A10 (Fig. 4 *A*). Under reducing conditions there was a major band with apparent $M_r$ of ~185 × $10^3$ and a minor band at ~175 × $10^3$. Under nonreducing conditions, the apparent $M_r$ of each band was decreased by ~15 × $10^3$ indicating the presence of one or more intrachain disulphide bonds.

After $^{125}I$ surface labeling of mouse serum-induced TPM, mAb SER-4 specifically immunoprecipitated a single species of Ag by SDS-PAGE, with an apparent $M_r$ of ~185 × $10^3$ (reduced) (Fig. 4 *B*) or ~170 × $10^3$ (nonreduced) (not shown). Since the lower $M_r$ species observed after metabolic labeling was not detected with surface labeling, it is likely to be an incompletely processed form of the molecule that is absent from the plasma membrane.

To determine whether the SER-4 Ag was one of the forms of the LC Ag (24) we carried out immunoprecipitations with a pan anti-LC mAb, M1/9.3 (12). Following surface labeling with $^{125}I$, the LC Ag ran under reducing conditions on SDS-PAGE as a broad band with a $M_r$ ranging from 155 to 180 × $10^3$. This was clearly distinct
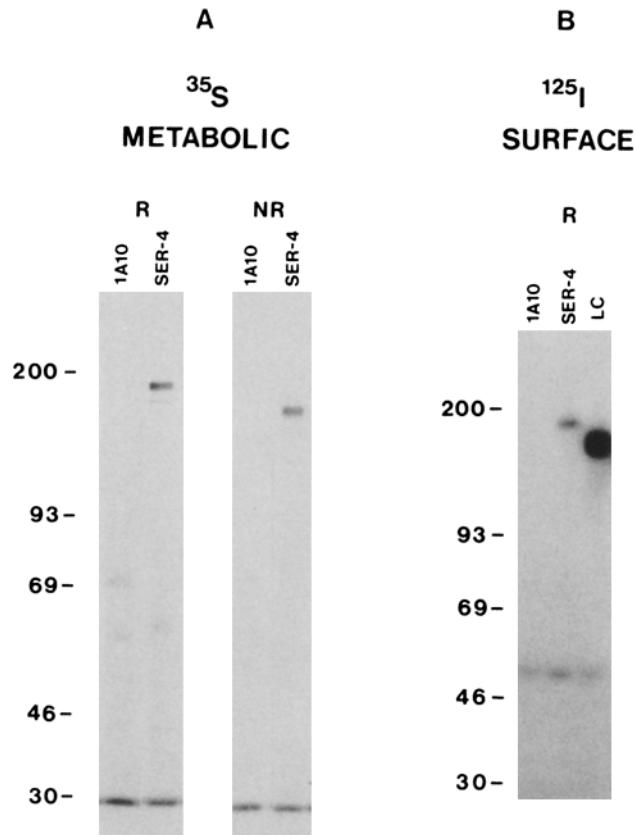
A

$^{35}S$

METABOLIC

B

$^{125}I$

SURFACE



FIGURE 4. 6.5% SDS-PAGE of (A) [$^{35}$S]methionine and [$^{35}$S]-cysteine-labeled TPM cultured in 15% mouse serum and (B) $^{125}$I-labeled TPM cultured in 15% mouse serum. After solubilization in 2% NP-40, immunoprecipitations were carried out with control mAb, 1A10, or with mAb SER-4 under reducing (R) or non-reducing (NR) conditions. The nonspecific band observed at 50 $M_r$ after surface iodination represents heavy chain of mouse Ig. IgG is adsorbed from mouse serum to TPM during culture, becomes iodinated, and subsequently binds protein A-Sepharose during immunoprecipitation. The positions of molecular weight standards ($\times$ 10$^{-3}$) are indicated.

from the SER-4 Ag immunoprecipitated from the same lysate and run on the same gel (Fig. 4 B). This demonstrates that the SER-4 Ag is not a member of the LC Ag family.

*Distribution of SER-4 Ag In Vivo.* In a previous study it was shown that expression of SER, assessed by rosette analysis, was restricted to certain subpopulations of stromal tissue MØ, especially those isolated from hematopoietic and lymphoid tissues. It was therefore important to compare the pattern of SER expression with the immunocytochemical distribution of SER-4 Ag in various tissues.

First, in cryostat sections of bone marrow plugs, mAb SER-4 revealed an arborizing network of stromal cells that were evenly distributed throughout the tissue (Fig. 5 A). These cells had the morphological features of resident bone marrow MØ (1, 6-8), and were intimately associated with hematopoietic cells. Immunofluorescence double-labeling experiments with the MØ-specific mAb, F4/80 (18), confirmed that SER-4⁺ cells were also F4/80⁺ (not shown). However, bone marrow monocytes, which label with F4/80, were not stained by SER-4. The selectivity of mAb SER-4 for the resident MØ population is consistent with previous rosetting experiments in which the resident MØ were shown to be the only bone marrow cells, including monocytes, able to bind sheep erythrocytes (9).
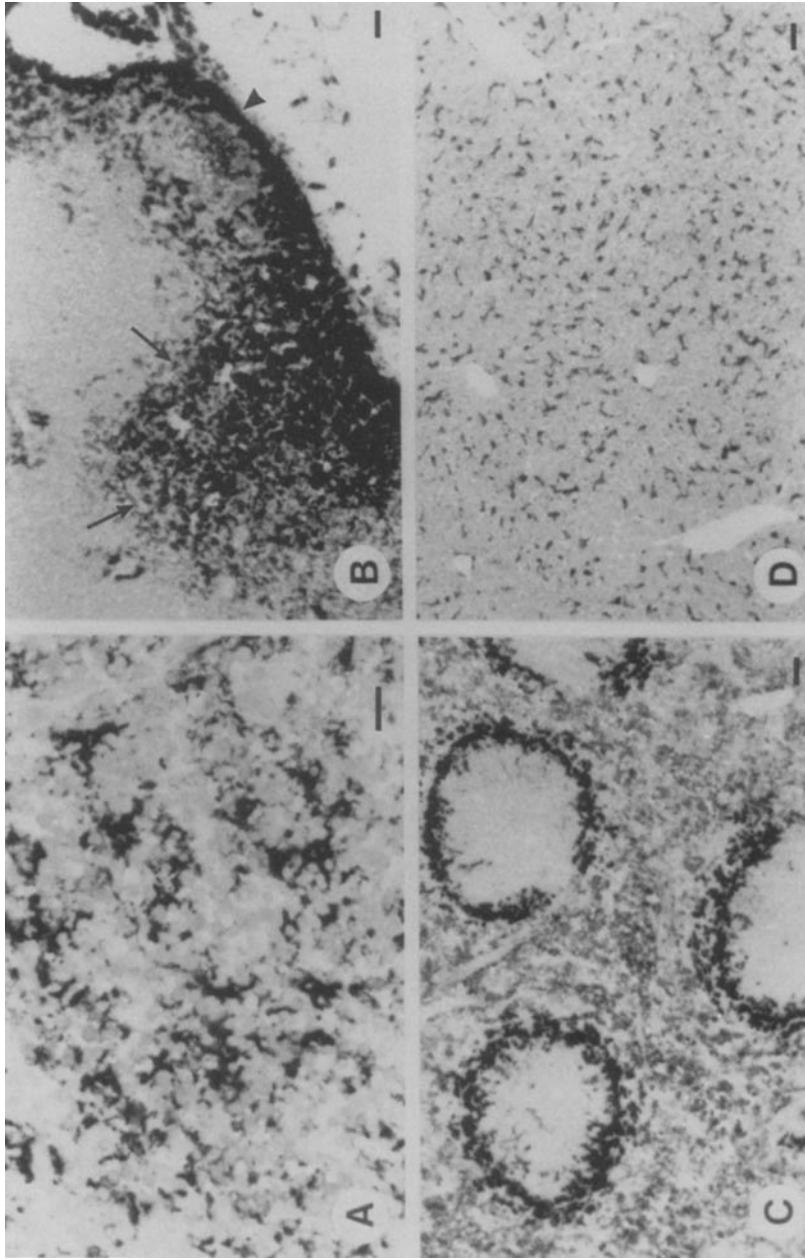
FIGURE 5. Photomicrographs of SER-4 Ag expression in situ. 7-$\mu$M cryostat sections were fixed in acetone and stained with the avidin-biotin immuno-peroxidase method. (A) Bone marrow plug showing network of SER-4$^+$ plasma membrane processes of the resident bone marrow M$\emptyset$ population. Monocytes and other developing hematopoietic cells are negative. (B) Mesen-teric lymph node showing intense staining of stromal M$\emptyset$ in subcapsular sinus (arrowhead) and medullary cords (arrows). (C) Spleen showing intensely positive SER-4$^+$ marginal metallophils at periphery of white pulp (arrows), with weaker staining on M$\emptyset$ in the marginal zone and red pulp. (D) Liver showing SER-4$^+$ sinus-lining Kupffer cells. Bars, 25 $\mu$M.

In lymph nodes, either mesenteric (Fig. 5 *B*), cervical or popliteal (not shown), intense staining was observed on stromal MØ in the subcapsular sinus and medullary cords, but tingible body MØ present in follicles were not stained. In some follicles, however, SER-4 revealed a reticular staining pattern within the developing germinal centers. The anatomical location and appearance of this staining pattern suggest that follicular dendritic cells may also be SER-4^{+} (25).

In the spleen, strong reactivity was observed on very stellate cells within the inner region of the marginal zone (Fig. 5 *C*). Weaker, but easily detectable staining was also present on marginal zone and red pulp MØ (Fig. 5 *C*). Examination under high power showed that the most strongly staining cells were on the follicular side of the marginal sinus. They can therefore be identified as marginal metallophils, a specialized subpopulation of spleen MØ (26, 27). In support of this conclusion, we found that MOMA-1, a rat mAb that specifically recognizes mouse marginal metallophils (13), produced a staining pattern in the spleen that was indistinguishable from that of SER-4 (not shown). In contrast to the other lymphoid tissues, the thymus showed no detectable staining with SER-4 (not shown).

In the liver, Kupffer cells were found to stain weakly, but uniformly with SER-4 (Fig. 5 *D*). In the lung, the stellate interstitial MØ stained strongly with SER-4 and
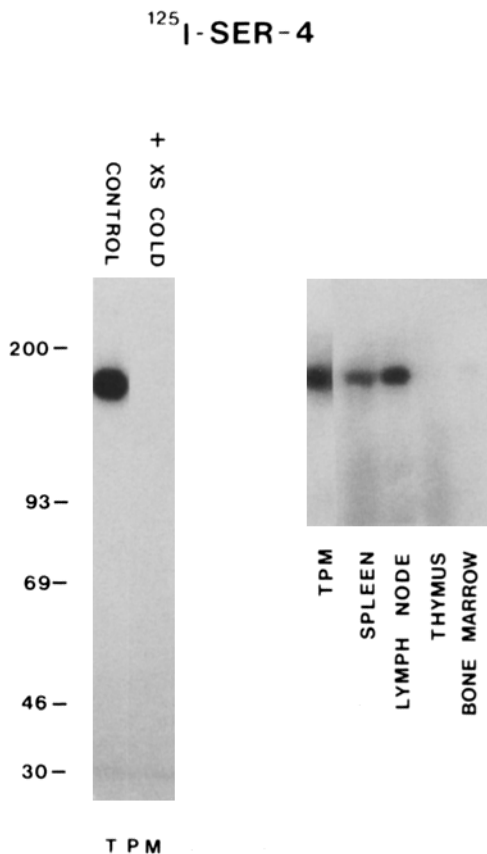


FIGURE 6. Western blots of SER-4 Ag expression in nonreduced lysates of mouse serum-induced TPM, spleen, lymph nodes, thymus, and bone marrow. Blots were prepared from 6.5% gels and probed with ^{125}I-SER-4 IgG in the absence or presence (*left*) panel of 100-fold excess unlabeled SER-4 IgG. A specific band at ~170 × $10^3$ $M_r$ can be seen in lysates of mouse serum-induced TPM, spleen, lymph node, and bone marrow, but not thymus. The weak signal observed in bone marrow lysates reflects the fact that lysates were normalized for organ input, rather than cell number. The positions of molecular weight standards ($\times 10^{-3}$) are indicated.

a subpopulation of bronchoalveolar M∅ was also positive (not shown). In the skin, no reactivity was observed on epidermal Langerhans cells, though scattered dermal histiocytes were strongly positive (not shown). In the small intestine, occasional M∅ in the lamina propria were stained and stromal M∅ underlying the dome epithelium of Peyer's patches were also positive (not shown). In the kidney, virtually no M∅ or other cell populations reacted with SER-4 (not shown). Finally, in the brain, microglia in the white and grey matter were completely negative for SER-4. Interestingly, however, the majority of M∅ outside the blood brain barrier (28), in the choroid plexus, pituitary gland, subfornical organ, and leptomeninges, were clearly positive (not shown).

*Nature of SER-4 Ag in Tissues.* Western blot analyses were carried out to determine the nature of the Ag recognized by mAb SER-4 in hematopoietic and lymphoid tissues (Fig. 6). With nonreduced lysates of mouse serum-induced TPM, used as a control, $^{125}$I-SER-4 IgG specifically bound a 170-kD molecule (Fig. 6). Indistinguishable bands at 170 kD were seen for spleen, lymph node, and bone marrow, but no band was visible in the thymus (Fig. 6). The rank order (normalized on an organ, rather than cell number basis) was lymph node > spleen > bone marrow. The relative amount of SER-4 Ag for each tissue is therefore in agreement with the overall intensity of staining observed by immunocytochemistry.

## Discussion

In this study we described the isolation and characterization of an mAb directed to SER, a novel M∅ receptor that binds unopsonized sheep E via recognition of sialylated glycoconjugates (9). Our strategy, to screen for mAbs that inhibit receptor function, is a powerful selection method that has been used previously to isolate mAbs to FcR, fibronectin receptor, and other adhesion receptors on M∅ (19, 29, 30).

Several lines of evidence indicate that the molecule recognized by mAb SER-4 is equivalent to SER, defined previously by rosetting studies. First, the time course of expression of the Ag on peritoneal M∅ cultured in mouse serum closely parallels that of SER. Second, the distribution of the Ag in situ is consistent with previous rosetting studies of SER expression on isolated tissue M∅ populations (9). Third, recent experiments in our laboratory indicate that the purified molecule specifically agglutinates sheep E (Crocker, P., unpublished observations).

It is likely that mAb SER-4 is directed close to, but not at, the putative sialic acid binding site of the receptor, since only 50% inhibition was obtained with F(ab')$_2$ fragments while Fab fragments had no effect on SER function, despite binding effectively to the SER-4 Ag. This pattern of inhibition is compatible with steric hindrance such that the degree of inhibition is proportional to the size of the mAb fragment. However, we cannot exclude a partial role for M∅ Fc receptors in mediating the inhibition by intact IgG.

The molecular properties and tissue distribution of the SER-4 Ag suggest that it is a previously uncharacterized differentiation Ag of mononuclear phagocytes. Immunoprecipitation and Western blotting experiments demonstrated that the SER-4 Ag is synthesized by M∅ as a single-chain plasma membrane protein that on SDS-PAGE has an apparent $M_r$ of $185 \times 10^3$ under reducing conditions and $170 \times 10^3$ under nonreducing conditions. Despite the similarity in $M_r$ to the LC Ag family of molecules, the SER-4 Ag was shown to be distinct from members of this family expressed on the same M∅ (24). The SER-4 Ag is of similar $M_r$ to several other

single chain lectin-like receptors present in eukaryotic cells. These include the mannose receptor (175 kD) and the cation-independent mannose-6-phosphate receptor (215 kD), both of which are expressed on M$\emptyset$ as well as other cell types (31–33). However, SER can be distinguished from both molecules on the bases of ligand specificity, behavior on SDS-PAGE, and tissue distribution (31–33).

The SER-4 Ag was found to be present at high levels on subpopulations of stromal tissue macrophages. The pattern of expression was in agreement with earlier sheep E rosetting assays for SER (9). Thus, in the bone marrow, SER-4 Ag was restricted to the resident bone marrow M$\emptyset$ population, with none being detected on the developing F4/80$^+$ monocytes. Particularly striking was the high level of expression by the majority of stromal M$\emptyset$ in lymph nodes that also rosette intensely with sheep E (9). The spleen showed a more complex pattern of reactivity for SER-4, with high levels observed only on marginal metallophils. Lesser, but clear staining was detected on M$\emptyset$ in the red pulp and the outer marginal zone. Since the majority of stromal M$\emptyset$ isolated from the spleen by collagenase digestion are from the red pulp and marginal zone (34, 35), the immunocytochemical staining pattern observed in the present study is consistent with previous rosetting analyses in which isolated stromal spleen M$\emptyset$ expressed moderate levels of SER (9).

Site number analysis of SER expression by TPM cultured in mouse serum showed that an average of up to $10^6$ molecules of SER-4 Ag were expressed per cell. Although $\sim$50% of this is intracellular (Crocker, P., unpublished observations), it is clear that when compared with other well-characterized plasma membrane receptors such as mannose receptor, CR3 and FcR, SER is potentially one of the most highly expressed M$\emptyset$-restricted surface molecules described to date.

How can the heterogeneity of SER expression on M$\emptyset$ populations be explained? We have recently demonstrated that the induction and maintenance of SER on M$\emptyset$ in vitro is regulated in a dose-dependent manner by a species-restricted activity in mouse plasma and lymph (11). The activity in plasma had an apparent $M_r$ of $\sim$70 $\times$ $10^3$ by gel filtration and this led us to propose that the heterogeneity of SER expression on different M$\emptyset$ populations could in part be explained by the relative concentration of inducing activity in different tissue compartments. In the present study, clear evidence for this possibility was seen in the central nervous system, where microglia within the blood brain barrier expressed no detectable SER-4 Ag, whereas M$\emptyset$ outside the blood brain barrier, in the choroid plexus and subfornical organ, expressed relatively high levels (28). Likewise, the absence of the SER-4 Ag on thymic M$\emptyset$ may be related to the presence of nonleaky endothelium in the cortex of this organ, creating a "blood-thymus barrier" (36).

It is clear, however, that there are additional mechanisms that regulate the level of the SER-4 Ag, because sinus-lining M$\emptyset$ in liver and spleen express intermediate levels, although they are continuously exposed to plasma. Since these M$\emptyset$ populations are important in the removal of effete E and since circulating mouse E express a sialylated ligand for SER (Crocker, P., unpublished observations), ingestion of effete E by these M$\emptyset$ could result in internalization and degradation of the SER-4 Ag.

The availability of an inhibitory mAb to SER will be a valuable tool in future studies investigating the functional significance of the receptor on M$\emptyset$ populations. The distribution of SER on selected populations of stromal tissue M$\emptyset$ is consistent with a role in cell-cell or cell-matrix interactions. Unlike other well-defined M$\emptyset$ lectin-like receptors, such as the mannose and galactose receptors (37, 38), SER does not

mediate phagocytosis of ligand-bearing particles and its primary function is therefore unlikely to be related to scavenging. If SER is involved in adhesive interactions in tissues such as bone marrow and lymph nodes, it should be possible to identify ligands on cells that are naturally associated with SER-4$^+$ stromal M$\emptyset$. Direct support for this possibility has come from our recent studies in which subpopulations of mouse bone marrow cells were shown to bind stably to SER$^+$ M$\emptyset$ in a manner indistinguishable from that of sheep E (Crocker, P., unpublished observations). In lymph nodes, SER-4$^+$ M$\emptyset$ in subcapsular sinuses and medullary cords interact with recirculating lymphocytes and plasma cells, respectively (25), and marginal metallophils of the spleen are thought to bind avidly to B lymphocytes (27).

It is therefore possible that SER contributes to local adhesive interactions that influence the movement of bone marrow cells and recirculation of lymphocytes. In addition, the finding that sialic acid binding proteins can be mitogenic for B and T lymphocytes raises the interesting possibility that SER is involved in growth regulation of attached cells via direct cellular signaling (39, 40).

## Summary

An inhibitory rat mAb, SER-4, has been raised to the mouse macrophage (M$\emptyset$)-restricted hemagglutinin, sheep erythrocyte receptor (SER), which binds unopsonized sheep erythrocytes through recognition of sialylated glycoconjugates. This receptor was originally defined on mouse resident bone marrow M$\emptyset$ where it was implicated in adhesive interactions of these cells with proliferating hematopoietic cells. In the present study using mouse serum–induced thioglycollate-elicited peritoneal M$\emptyset$ (TPM) as a model system for SER expression, mAb SER-4 IgG2a completely blocked rosette formation at 1 $\mu$g/ml. The inhibition was likely to be via steric hindrance rather than through a direct interaction with the putative sialic acid binding site of SER because F(ab')$_2$ and Fab fragments of mAb SER-4 gave a maximum inhibition of 50–60% and 0% respectively, despite binding effectively to the SER-4 antigen (Ag). Immunoprecipitation and Western blotting experiments with cultured M$\emptyset$ or tissue extracts demonstrated that the Ag recognized by SER-4 mAb is a single chain molecule with an apparent $M_r$ by SDS-PAGE of 185 $\times$ 10$^3$ (reduced) or 170 $\times$ 10$^3$ (non-reduced) and is distinct from members of the leukocyte common Ag family. Expression of SER and SER-4 Ag in culture were closely correlated and depended on the presence of mouse serum for optimal induction. Further evidence that the SER-4 Ag is functionally equivalent to SER was provided by immunocytochemistry in which the overall pattern of staining in tissues was consistent with previous rosetting experiments. In the bone marrow, expression of the SER-4 Ag was restricted to the resident bone marrow M$\emptyset$ population with no expression on monocytes. High expression was also observed on stromal M$\emptyset$ within the subcapsular sinus and medullary cords in lymph nodes and on marginal metallophils in the spleen. These results therefore confirm that SER is a novel M$\emptyset$-restricted receptor whose distribution and properties indicate a role in cellular interactions in hematopoietic and lymphoid tissues.

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