0022-1767/82/1283-1221$02.00/0
THE JOURNAL OF IMMUNOLOGY
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Vol. 128, No. 3, March 1982
Printed in U.S.A.

MAC-2, A NOVEL 32,000 M_r MOUSE MACROPHAGE SUBPOPULATION-SPECIFIC ANTIGEN DEFINED BY MONOCLONAL ANTIBODIES[1]

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Two monoclonal antibodies, M3/31 and M3/38, were obtained by fusion of mouse myeloma cells with rat spleen cells immunized to immunoadsorbent-purified macrophage glycoproteins. Co-precipitation experiments show that antigenic determinants recognized by these two antibodies reside on the same molecular species, termed Mac-2. Mac-2, an antigen of 32,000 M_r, is synthesized by and expressed on the surface of thioglycollate-elicited macrophages as shown by [^{35}S]-methionine and ^{125}I labeling. Saturation binding experiments show that thioglycollate-elicited macrophages express 1.7 × 10^5 Mac-2 sites/cell. Thioglycollate-elicited macrophages are strongly absorptive for ^{125}I-labeled M3/38 MAb. Kidneys are also absorptive; however, evidence is presented pointing to the nonspecificity of this absorption. Lymph node and thymus are negative, whereas spleen and bone marrow are weakly absorptive, probably due to stromal cells. Nonlymphoid tissues, such as lung, liver, heart, and brain, exhibit slight or no absorbing capacity. Cell suspensions from spleen, bone marrow, thymus, and peripheral lymph node are >99% Mac-2$^-$ by immunofluorescent flow cytometry. In contrast, thioglycollate-elicited macrophages are >96% strongly positive for Mac-2. Only 20% of peptone-elicited cells are weakly positive, whereas resident peritoneal macrophages and other macrophages elicited by *Listeria monocytogenes*, Con A, or LPS are >98% negative. SDS-PAGE of [^{35}S]-methionine-labeled Mac-2 shows that thioglycollate-elicited macrophages synthesize 10- to 30-fold more Mac-2 than other peritoneal macrophage subpopulations, whereas all types of peritoneal macrophages synthesize and express on their surfaces similar amounts of the Mac-1 antigen. Mac-2 antigen is therefore induced in macrophages only in response to specific differentiative signals.

Mononuclear phagocytes residing in different anatomical sites are derived from common precursors in the bone marrow. However, they differ markedly in their morphology, metabolism, surface receptors, enzyme content, and functional properties (1-4). For example, bone marrow or peripheral blood monocytes possess large amounts of peroxidase in granules, whereas resident peritoneal macrophages, Kupffer cells, and alveolar macrophages have only low levels of this enzyme (1). Moreover, the number of complement (C) receptors in peripheral blood monocytes and peritoneal macrophages is higher than that of other phagocytes in the liver, lung, and bone marrow (1). Striking differences are also seen between resident peritoneal macrophages and those elicited by different agents. In general, elicited macrophages have increased levels of plasminogen activator but diminished levels of 5'-nucleotidase. They phagocytize C-coated red cells readily, whereas resident macrophages do not (5, 6). However, great heterogeneity also exists among peritoneal macrophages elicited in response to different stimuli. Hence, peritoneal macrophages elicited by *Corynebacterium parvum* but not by thioglycollate (TG)[3] are tumoricidal, (6, 7), and these populations also exhibit distinct ectoenzyme profiles (6, 8). Furthermore, the ability of lymphokines to induce interferon production by peritoneal macrophages varies with the mechanism of elicitation (9).

To understand the development and phenotypic divergence of these macrophage subpopulations, antibodies to distinct subpopulations have been developed. For example, an absorbed antiserum reactive with tumoricidal peritoneal macrophages activated by *C. parvum* or pyran but not with nontumoricidal glycogen-elicited cells has been described (10).

Previous work from this laboratory described Mac-1, a murine phagocyte differentiation antigen identified by the monoclonal antibody M1/70 (11). The antibody cross-reacts with an antigen with a similar distribution on human cells (12). Mac-1 is composed of two noncovalently associated polypeptides of 190,000 and 105,000 molecular mass (M_r). It is expressed in large quantities on TG-induced macrophages and in lesser amounts on peripheral blood monocytes, granulocytes, bone marrow myeloid cells, and natural killer cells (13). Moreover, recent studies indicate that essentially all peritoneal macrophages elicited by bacteria, nonspecific inflammatory agents, or immunomodulators such as Con A and lipopolysaccharide (LPS) express Mac-1 (14).

We have previously described a novel cascade procedure that was used to identify further antigens on the macrophage cell surface (15). TG-induced mouse peritoneal exudate cell (PEC) membranes were detergent solubilized, and the glycoprotein fraction was obtained by *Lens culinaris* lectin affinity chromatography. Two previously defined antigens shared between lymphocytes and PEC were removed with monoclonal antibody (MAb) immunoadsorbents. Rats were primed with this purified, immunodepleted antigen preparation, and their spleen cells fused to the NSI myeloma. Hybridoma cultures were obtained that precipitated macrophage surface antigens of 32,000 M_r, 110,000 M_r, and 180,000 M_r.

In this communication, we report the biochemical characterization and cell distribution of the 32,000 M_r polypeptide,

Received for publication September 21, 1981.
Accepted for publication November 20, 1981.
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[1] This work was supported by United States Public Health Service Grant CA-27547 and a Grant from The Council for Tobacco Research.
[2] Supported by postdoctoral fellowships from The Anna Fuller Fund and the National Institute for Allergy and Infectious Diseases.

[3] Abbreviations used in this paper: DME, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MAb, monoclonal antibody; M_r, molecular mass; PBS, 0.01 M $NaPO_4$, 0.14 M NaCl, pH 7.3; PEC, peritoneal exudate cells; TG, thioglycollate.

termed Mac-2. In contrast to Mac-1, the expression of Mac-2 is preferentially associated with peritoneal macrophages elicited by TG. Unelicited peritoneal macrophages and macrophages elicited by protease peptone, Con A, LPS, and *Listeria monocytogenes* are either weakly positive or negative. Therefore, Mac-2 expression is induced only by strong inflammatory stimuli and appears specific for mononuclear phagocyte subpopulations in a distinct stage of differentiation.

MATERIALS AND METHODS

Hybridoma lines and MAb. The characteristics of the antibodies used in this paper are summarized in Table I. The derivation of subcloned lines of M1/69, M1/70 (11, 16), M1/42, and M1/84 (17) has been previously described. M3/31 and M3/38 were obtained by fusion of NSI myeloma cells (Cell Distribution Center, Salk Institute, San Diego, CA) with rat spleen cells immunized to TG-induced macrophage glycoproteins that had been depleted of previously identified antigens with monoclonal antibody immunoadsorbents (15). These lines were subcloned twice to achieve stability. Cells were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 10% horse serum. Clones are designated numerically; e.g., M3/31.1 and M3/31.1.1 represent a clone and subclone of M3/31, respectively. For brevity, such designations are usually omitted in the text.

To obtain large quantities of purified MAb, hybridoma lines are grown in DME containing 5% fetal calf serum (FCS) in 1 liter spinner cultures. MAb were purified by $(NH_4)_2SO_4$ precipitation and DEAE and Sephadex G-200 chromatography as previously described (12).

Selection and analysis of HL and HK variants. M3/31.1.1.6 HLK and M3/38.1.2 HLK lines were plated in soft agar at 1×10^3, 3×10^3, and 9×10^3 cells/100-mm petri dish as described (16). HL variant screening was as previously described in the note added in proof of Reference (18). HK variants were screened for absence of reactivity with rabbit anti-rat Fab in Mancini radial immunodiffusion as described (18). For confirmation of putative HL and HK clones, supernatants internally labeled with 0.05 μCi [^{14}C]-leucine (New England Nuclear, 342 mCi/mM) were analyzed by SDS-PAGE (16).

Cell preparation. Male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), 8 to 16 wk of age, were used in most experiments. Peritoneal exudates were obtained after i.p. injection of one of the following reagents: 1.5 ml Brewer's TG, 1.5 ml 10% protease peptone, 40 μg *Salmonella typhosa* LPS (Westphal) (all three reagents were from Difco Laboratories, Detroit, MI), 15 μg Con A (Sigma Chemicals, St. Louis, MO), or 2×10^4 live *Listeria monocytogenes* organisms (the kind gift of Dr. Emil Unanue). The time of treatment was usually 4 days for TG, and 3 days for the others. Cells were harvested in PBS[3] containing 20 U/ml heparin (Sigma). For immunofluorescent studies of glass-adherent macrophages, cells were resuspended to 2×10^5/ml in RPMI 1640 supplemented with 10% FCS and 10 mM HEPES.[3] Cells (1 ml) were incubated in 16-mm culture wells (Costar, Cambridge, MA) containing a 12-mm round coverslip (Bellco Glass, Vineland, NJ) for 2 hr at 37°C. They were then processed according to the procedures of Beller *et al.* (19).

Site number estimation. Purified M3/38.1.2 HL2 (0.1 mg/ml) was iodinated with 0.5 to 1 mol carrier-free ^{125}I per mol protein using 1,3,4,6-tetrachloro-3,6-diphenylglycouril and the methods of Fraker and Speck (20), except that the reaction was terminated by transfer to fresh tubes containing 20 μl of 0.4 mg/ml L-tyrosine.

TABLE I
Characteristics of monoclonal antibodies used in this paper

Antibody	Chain Composition[a]	Subclass	Concentration	Antigen: Designation	Antigen: Polypeptide chain M_r	References
			μg/ml			
M1/42.3.9.8	HLK	IgG2a	106	H-2	48,000 46,000 12,000	17
M1/69.16.11 HK 2	HK	IgG2b	ND[b]	None[c]		16, 18
M1/70.15.1	HL	IgG2b	100	Mac-1	190,000 105,000	11, 16
M1/84.1.8	HLK	IgG2a	52	Not named	46,000	17
M3/31.1.1.6	HLK	IgM	40	Mac-2	32,000	15[d]
M3/38.1.2	HLK	IgG2a	110	Mac-2	32,000	15[d]
M3/38.1.2 HL 2	HL	IgG2a	280	Mac-2	32,000	[d]

[a] H, specific heavy; L, specific light; K, myeloma kappa.
[b] Not done.
[c] No specific binding due to the loss of specific light chain synthesis.
[d] Described in this paper.

TG-elicited PEC (20 μl, 10^8/ml) were mixed with 80 μl of ^{125}I M3/38.1.2 HL2 at varying concentrations. The amount of binding was determined after shaking 1 hr at 4°C as described under "Tissue absorption studies."

Tissue absorption studies. Lung, liver, brain, heart, and kidney from freshly sacrificed animals were homogenized between the frosted ends of two glass slides. Large fragments were removed by 1 × G sedimentation for 3 min, and the homogenate was washed twice in PBS by centrifugation at 2000 × G for 15 min. Lymphoid organs, such as spleen, thymus, peripheral lymph node, and bone marrow from femurs were teased with forceps. The resulting cells and residual tissue stroma were washed twice in PBS before use. All procedures were carried out at 4°C. Aliquots of tissues were assayed for protein by the method of Lowry *et al.* (21).

Limiting concentrations of ^{125}I M3/38 HL (determined by titrating a constant number of cells with increasing concentrations of antibody) were mixed with equal volumes of fivefold diluted tissues in 96-well microtiter plates. PBS was used as the negative tissue control. After shaking at 4°C for 1 hr and centrifuging at 2000 × G for 15 min, the residual antibodies were recovered from the supernatant. Ten microliters of the absorbed antibody were added to 1×10^5 TG-elicited PEC in 50 μl PBS, 10% BSA, and 4 mg/ml human IgG. After shaking for 1 hr at 4°C, the cells were washed four times with 0.01 M Tris-HCl, pH 7.4, 0.14 M NaCl, and 1% horse serum and were gamma counted.

Immunofluorescent flow cytometry. Cells (50 μl, 2×10^7/ml) were incubated with equal volumes of MAb-containing spent culture supernatants for 30 min, or with control M1/69 HK supernatant or NSI supernatant plus 50 μg/ml normal rat IgG. The cells were washed 3 times and resuspended in 50 μl of affinity-purified fluorescein isothiocyanate- (FITC) rabbit $F(ab')_2$ anti-rat IgG, absorbed with mouse IgG, for 30 min. After another three washes, the cells were analyzed on a Becton-Dickinson fluorescence-activated cell sorter (FACS II) with glutaraldehyde-fixed sheep red cells used as standards. All procedures were carried out in L15 medium + 10 mM HEPES + 11 mM glucose at 4°C. During the second-stage incubation and subsequent washes, 0.01 M sodium azide was included in the medium to prevent patching.

Immunoprecipitation. Cells were surface labeled with ^{125}I by a modification of the lactoperoxidase-glucose oxidase method (22). For biosynthetic labeling, 10^7 PEC were plated on tissue culture flasks and incubated 18 hr at 37°C unless otherwise specified. Nonadherent cells were washed off, and adherent cells were labeled with 200 μCi L-[^{35}S]-methionine (New England Nuclear) in 1 ml of methionine-free DME for 6 hr. Subsequently, the adherent monolayer was washed three times in PBS and detergent solubilized. After centrifugation at 100,000 × G for 1 hr, the supernatant was recovered. Immunoprecipitation and SDS-PAGE were carried out as described (16).

RESULTS

Characterization of the antibodies secreted by M3/31, M3/38, and variant subclones. Supernatants from two stable cell lines, M3/31 and M3/38, were found to precipitate a 32,000 M_r polypeptide from ^{125}I-labeled PEC. M3/31.1.1.6 secretes an H chain with μ-like mobility in SDS-PAGE as well as specific L and myeloma K chains (Fig. 1) and types as an IgM in double immunodiffusion with subclass-specific reagents. M3/38.1.2 secretes a γ-like H chain and L and K chains (Fig. 1) and types as an IgG2a. To obtain homogeneous HL antibody for use in direct binding and site number quantitation experiments (see below), over 470 subclones of each line were screened for K chain loss variants as described in *Materials and Methods.* Two HL variants were isolated from M3/38, whereas only HK and L variants (the latter resulting from heavy chain loss) were obtained from M3/31 (Fig. 1). The specificity of the interaction of M3/31 with macrophage surfaces was demonstrated by the requirement of both specific H and L chains for activity in an indirect binding assay (Fig. 1).

Biochemical characterization of Mac-2 antigen. Both M3/31 and M3/38 precipitated a 32,000 M_r and lesser amounts of a 30,000 M_r polypeptide from ^{125}I-labeled PEC (Fig. 2*A*). Smaller amounts of a 60,000 M_r polypeptide have also sometimes been detected only after ^{125}I labeling. Mac-2 antigens do not contain interchain disulfide bonds, because samples treated with either reducing agents or iodoacetamide had identical mobilities in SDS-PAGE (Fig. 2*A* and *C*). Immunoprecipitation of Mac-2 antigen was also attempted from spleen cells (Fig. 2). Previously published control experiments showed that similar

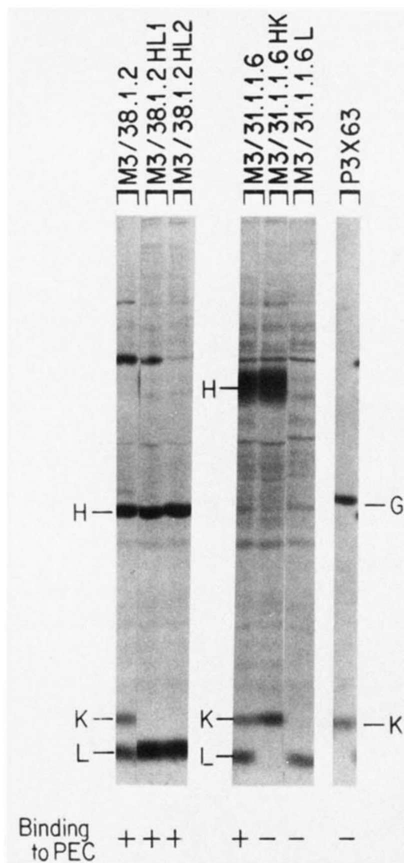

Figure 1. SDS-PAGE of [^{14}C]-labeled clonal supernatants of M3/31.1.1.6, M3/38.1.2, and their variants. Variants from M3/31.1.1.6 and M3/38.1.2 were isolated as described in *Materials and Methods* and labeled with [^{14}C]-leucine. After reduction in 5% 2-mercaptoethanol, 20 μl of supernatants were electrophoresed in 10% polyacrylamide gels and autoradiographed. To locate the position of myeloma K light chains, labeled supernatants from P3, parent line of the fusion partner NSI myeloma, were included. Binding of unlabeled supernatants to fresh 4-day TG-elicited PEC was determined in an indirect binding assay (16).

amounts of the common leukocyte antigen (CLA) were immunoprecipitated from the same spleen cell and PEC preparations (11). Extremely small amounts of Mac-2 antigen precipitated from spleen were detected only after prolonged autoradiogram exposure (Fig. 2*B*). This suggests that an antigen chemically similar to that on macrophages is expressed only in comparatively very small amounts on spleen cell surfaces.

To determine whether Mac-2 is biosynthesized by macrophages or acquired from an external source, macrophages were isolated from PEC by adherence and labeled with [^{35}S]-methionine. The 32,000 and 30,000 M_r polypeptides labeled with ^{125}I were also labeled with [^{35}S]-methionine, suggesting that they are both on the cell surface and biosynthesized by macrophages (Fig. 3). However, the 60,000 M_r polypeptide sometimes seen in immunoprecipitates of ^{125}I-labeled cell lysates is not seen when [^{35}S]-methionine-labeled cells are used. Comparatively similar amounts of Mac-2 and H-2 antigens are synthesized by adherent macrophages (Fig. 3). By exposing autoradiograms for varying lengths of time, the relative amount of the 32,000 and 30,000 M_r polypeptides was found to range from 4:1 to 25:1, depending on the batch of cell lysate used. This is the case both for [^{35}S]-methionine (Fig. 3) and ^{125}I labeling (not shown). Much smaller amounts of polypeptides of 27,000, 25,000, and 23,000 M_r are also precipitated by M3/38, but are not seen in M3/31 precipitates even after prolonged autoradiogram exposure (Fig. 3*B*). Thus, these smaller polypeptides appear to bear the M3/38 but not the M3/31 determinant.

The M3/31 and M3/38 target antigens are indistinguishable by the criterion of SDS-PAGE mobility. To test whether the M3/31 and M3/38 antigenic determinants were present on the same 32,000 and 30,000 M_r polypeptides or on different but electrophoretically indistinguishable molecules, co-precipitation experiments were carried out. Pre-precipitation with M3/38 removed material reactive with M3/31 (Fig. 4) and vice versa (data not shown). Therefore, the M3/31 and M3/38 antigenic determinants are present on the same molecular species.

Quantitation of Mac-2 sites on TG-elicited peritoneal macrophages. To estimate the number of Mac-2 sites per TG-elicited macrophage, cells were labeled with saturating amounts of ^{125}I-labeled M3/38 MAb (a representative experiment is shown in Fig. 5). In four independent experiments, an average of $1.7 \pm 0.2 \times 10^5$ molecules of ^{125}I M3/38 IgG were bound per TG-elicited macrophage. Binding was >95% inhibitable by unlabeled M3/38 MAb but not normal rat IgG (data not shown).

Distribution of Mac-2 on various tissues and cells: Tissue absorption studies. Entire homogenized lymphoid tissues, including the residual stroma to which macrophages are generally tightly adherent, were assessed for Mac-2 content by absorption experiments (Fig. 6*A*). Thymus and peripheral

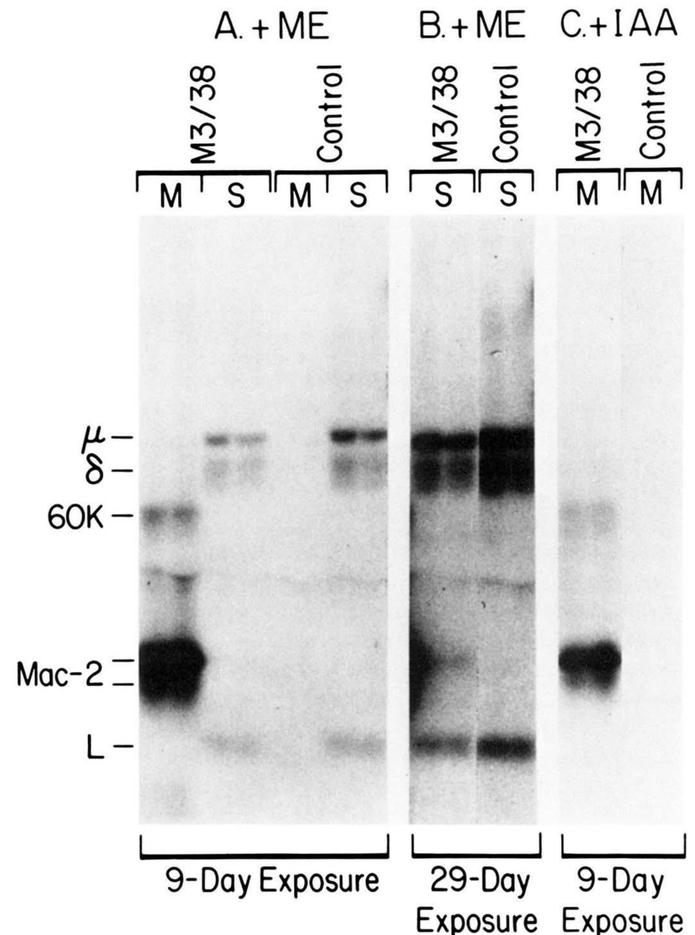

Figure 2. SDS-PAGE of immunoprecipitates from ^{125}I-labeled 4-day TG-elicited macrophages and spleen cells. Equal quantities (240,000 cpm) of macrophage (M) and spleen (S) detergent-solubilized cell lysates were indirectly precipitated by M3/38 or NSI supernatants containing 50 μg/ml normal rat IgG as control. Immunoprecipitates dissolved in reduced sample buffer (+ 5% 2-mercaptoethanol) (*A, B*) or nonreduced sample buffer (+ 50 mM iodoacetamide) (*C*) were electrophoresed in 5 to 12% polyacrylamide gradient gels. In addition to specific bands, surface Ig chains were also precipitated from spleen cells due to cross-reaction with the second layer reagent, rabbit anti-rat IgG.

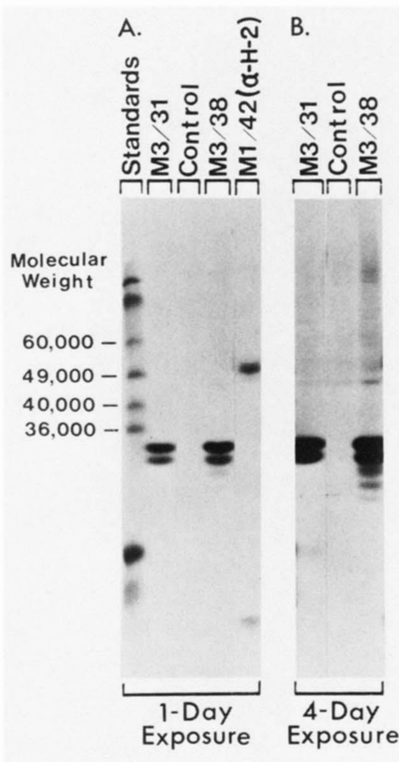

Figure 3. SDS-PAGE of [^{35}S]-methionine-labeled macrophage antigens precipitated by anti-Mac-2 (M3/31, M3/38), and anti-H-2 (M1/42) monoclonal antibodies. TG-elicited peritoneal exudate macrophages were purified by adherence and labeled with [^{35}S]-methionine as described in *Materials and Methods*. Triton X-100 cell lysates were precleared with pure rabbit anti-rat IgG conjugated to Sepharose CL-4B (10 mg antibody/ml beads), incubated 2 hr at 4°C with MAb culture supernatants (30 μl), then shaken for 1 ½ hr at 4°C with 5 μl of anti-rat IgG beads. Beads were washed, boiled in SDS sample buffer containing 5% 2-mercaptoethanol, and the eluate was subjected to SDS 5–15% gradient PAGE and fluorography (30). The control was NSI culture supernatant plus 50 μg/ml of normal rat IgG.

Figure 4. Coprecipitation of Mac-2 by M3/31 and M3/38. TG-induced PEC were surface-labeled with ^{125}I by using lactoperoxidase. Triton X-100 lysates were precleared with M3/38 or M1/69 (an irrelevant control) MAb followed by rabbit anti-rat IgG Sepharose as described in the legend to Figure 3. The resulting supernatants were then immunoprecipitated with 100 μl of M3/31 or M3/38 MAb and an excess of rabbit anti-rat IgG, and subjected to SDS 8 to 15% gradient PAGE and autoradiography.

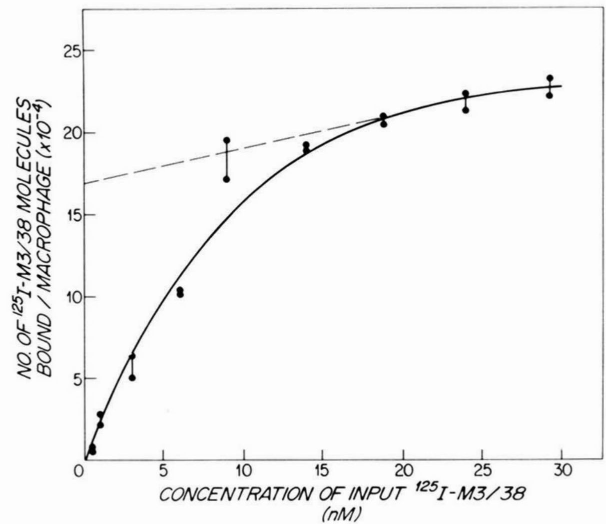

Figure 5. Saturation binding of ^{125}I-M3/38 to TG-elicited macrophages. Increasing concentrations of ^{125}I-M3/38 were incubated with 3 × 10^5 4-day thioglycollate-elicited PEC and binding was determined as described in *Materials and Methods*. Differential counts of Wright-Giemsa-stained PEC showed that approximately 85% were macrophages. Specific activity of ^{125}I-M3/38 MAb was 4.64 × 10^6 cpm/μg. The number of sites was determined by extrapolating to the y axis (dotted line), which corrects for nonsaturable nonspecific binding. By assaying the binding in presence of excess unlabeled M3/38, the nonspecific binding was found to range from 2 to 5%, depending on the concentration of input ^{125}I-M3/38.

lymph node were Mac-2 negative. In contrast, TG-induced PEC were strongly Mac-2 positive, whereas spleen and bone marrow contained about 10-fold less Mac-2 on a protein weight basis. Specificity was demonstrated by the lack of absorption by rat lymphoid tissues.

Among nonlymphoid tissues (Fig. 6*B*), heart and brain were negative, whereas lung and liver were slightly absorptive. Surprisingly, mouse kidneys appeared strongly absorptive. However, this was nonspecific, because the same results were obtained with rat kidneys. Furthermore, the kidneys did not truly absorb the M3/38 MAb, but left it in the supernatant in an inactive form (data not shown). In addition, frozen kidney sections showed no staining by M3/38 and FITC-rabbit anti-rat IgG.

Expression in hematopoietic and lymphoid tissues. The presence of Mac-2 on lymphoid single cell suspensions was examined by immunofluorescent flow cytometry. Mac-2 was expressed on essentially all macrophages in TG-induced peritoneal exudates (Fig. 7*d*). Smaller cells in these preparations, including polymorphs and lymphocytes, were negative for Mac-2 (Fig. 7*d*) but were stained by the M1/84 MAb, which has broad anti-leukocyte reactivity (Fig. 7*h*). In contrast to Mac-1, which is expressed on 40 to 50% of nucleated bone marrow cells (Fig. 7*e*), Mac-2 is expressed on less than 1% of bone marrow cells (Fig. 7*c*). Mac-2 is also expressed on <1% of cells from spleen, peripheral lymph node, and thymus (Table II).

Expression of Mac-2 on PEC induced by various agents. To determine whether Mac-2 is a subpopulation-specific macrophage antigen, peritoneal macrophages elicited by inflammatory agents, bacteria, and other stimuli were examined by immunofluorescence (Fig. 8). Mac-2 expression was found to be restricted to macrophages elicited by highly inflammatory agents. TG-elicited macrophages were strongly stained by M3/38 (>96% positive, Fig. 8*a*), whereas approximately 20% of peptone-elicited macrophages were only weakly positive (Fig. 8*b*). Greater than 98% of macrophages induced by Con A, LPS, or *Listeria monocytogenes*, and unelicited macrophages were negative (Fig. 8*c–f*). Moreover, granulocytes in 1-day TG-elicited exudate, which consist of mostly neutrophils, were weakly positive for Mac-1 (11) but negative for Mac-2 (Table II). In contrast, Mac-1 antigen was strongly expressed on >86% of the macrophages in all of these same preparations (14). Similar findings were obtained when glass-adherent cells from

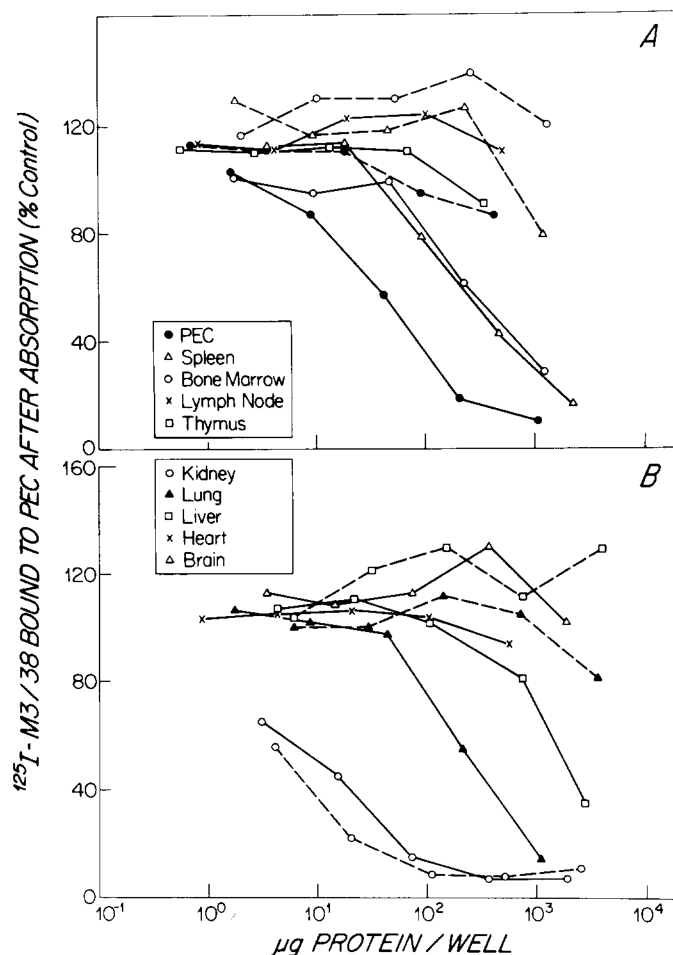

Figure 6. Absorption of ^{125}I-M3/38 with various mouse and rat tissues. Limiting amounts of ^{125}I-M3/38 were incubated with increasing quantities of mouse (——) or rat (-----) tissues (*A*, lymphoid; *B*, nonlymphoid). Residual binding activity to PEC was determined in a direct binding assay as described in *Materials and Methods*. Binding capacity was calculated as the percentage of ^{125}I-M3/38 bound after absorption with PBS.

these exudates were examined microscopically after indirect immunofluorescent staining (results not shown). Microscopic examination also confirmed that granulocytes in 1-day TG exudates were Mac-2 negative. These preparations also contained (20%) cells with morphology of macrophages, which were Mac-2 positive.

To confirm that expression of Mac-2 by macrophages was dependent on the eliciting agent, peritoneal macrophages elicited by various agents were purified by plastic adherence and internally labeled with [^{35}S]-methionine. Spleen and lymph node cells were also labeled. Cell lysates containing equal quantities of incorporated radioactivity were then analyzed by immunoprecipitation and SDS-PAGE. Macrophages elicited by TG for 1 or 4 days (Fig. 9, lanes 1 and 2) synthesized much higher quantities of Mac-2 than resident macrophages or macrophages elicited by Con A, LPS, *Listeria*, or peptone (Fig. 9, lanes 3 through 7). Comparison of fluorograms exposed for different lengths of time showed that the amount of Mac-2 synthesized ranged from 1/10 (for Con A-elicited macrophages) to 1/30 (for unelicited peritoneal macrophages) that of 4-day TG-elicited cells. In contrast, all these macrophages synthesized similar amounts of Mac-1 (14). Mac-2 was not synthesized by spleen or lymph node cells (Fig. 9, lanes 8 and 9).

DISCUSSION

Previous work from our laboratory described the use of a cascade procedure for MAb production and its application to the macrophage cell surface. Plasma membrane glycoproteins were purified by *Lens culinaris* lectin affinity chromatography and depleted of several immunodominant, nonmacrophage-specific antigens with MAb immmunoadsorbents. Rats were immunized with antigen prepared in this manner from TG-induced mouse macrophages. This approach allowed the identification with MAb of two previously undescribed macrophage antigens of 32,000 M_r and 110,000 M_r. In this report, we have described the cellular distribution and biochemical characterization of the 32,000 M_r antigen, termed Mac-2. Furthermore,

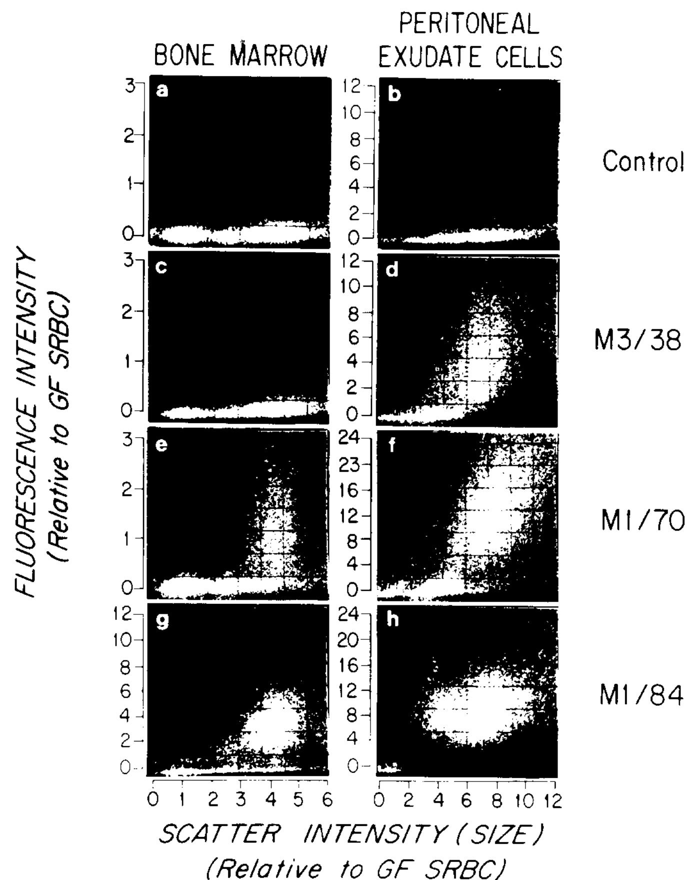

Figure 7. Immunofluorescence flow cytometry dot plot analysis of TG-elicited PEC and bone marrow cells. Dots represent fluorescence and scatter intensities of individual cells. Cells were first labeled with clonal supernatants or NSI supernatant containing 50 µg/ml normal rat IgG as control. The second stage reagent was FITC-rabbit F(ab')$_2$ anti-rat IgG previously absorbed with mouse IgG. Scales are plotted relative to the fluorescence and scatter intensities of glutaraldehyde-fixed sheep red blood cells (GF SRBC) which were arbitrarily assigned a value of unity. Lymphocytes, polymorphonuclear leukocytes, and macrophages appear at scatter intensities of approximately 2-3, 3-5, and 6-10, respectively.

TABLE II

Distribution of Mac-2 on various lymphoid cells

Cell Type	% Positive[a]
TG-macrophages (4 d. PEC)[b]	96
TG-granulocytes (1 d. PEC)[c]	<1
Spleen	<1
Bone marrow	<1
Thymus	<1
Lymph node	<1

[a] Determined by FACS analysis as described in *Materials and Methods*.
[b] Scatter gates set to exclude granulocytes and smaller cells.
[c] Scatter gates set to include granulocytes and exclude cells with size of macrophages.

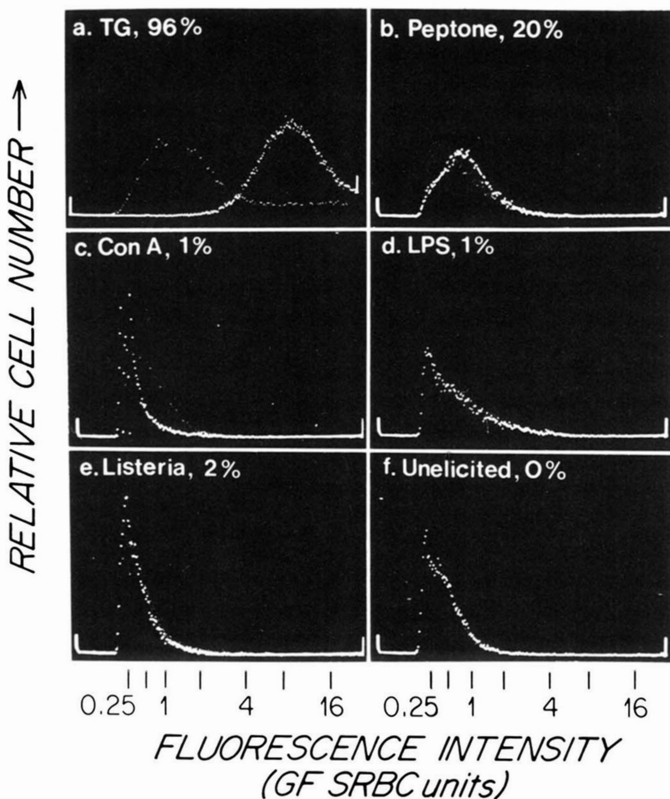

Figure 8. Immunofluorescence flow cytometry analysis of peritoneal macrophages elicited by different agents. PEC were labeled with M3/38.1.2 HL2 (*bright curve*) or NSI control supernatants plus 50 μg/ml normal rat IgG (*dim curve*) as described in *Materials and Methods*. The scatter gates were set to exclude RBC, dead cells, and most lymphocytes and polymorphonuclear leukocytes. The histograms shown are plotted with the relative cell number on a linear scale and fluorescence on a logarithmic scale.

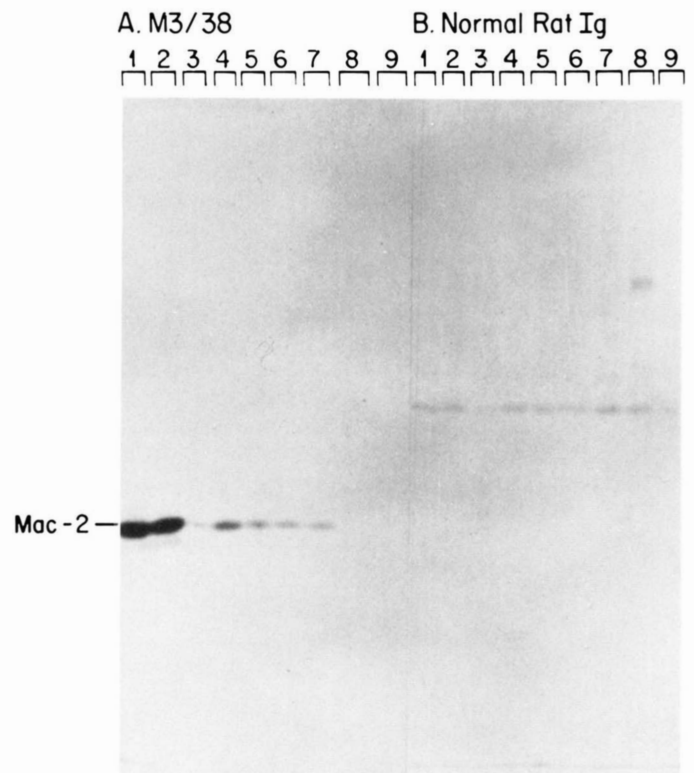

Figure 9. SDS-PAGE of [^{35}S]-methionine-labeled Mac-2 antigens from macrophages elicited by various means and spleen and lymph node cells. Resident or exudate macrophages were obtained as described in *Materials and Methods*, purified by 2-hr adherence on tissue culture flasks, and labeled with [^{35}S]-methionine as in *Materials and Methods*. Single cell suspensions from spleen and lymph node were processed similarly except that the adherence step was omitted. Equal quantities of Triton-X 100 lysates (1.5 × 10^6 cpm) were shaken with 8 μg of M3/38 1.2.8 HL2 coupled to Sepharose CL-4B for 90 min at 4°C. Controls were 8 μg of normal rat IgG coupled to Sepharose CL-4B. Beads were washed and boiled in SDS-sample buffer containing 5% 2-mercaptoethanol, and the eluates were subjected to SDS-10% PAGE and fluorography (30). The macrophage lysates used for immunoprecipitation were elicited as follows: *Lane 1*, 4-day TG; *2*, 1-day TG; *3*, unelicited; *4*, Con A; *5*, LPS; *6*, *L. monocytogenes*; *7*, protease peptone. Lysates of spleen and lymph node cells were used in lanes *8* and *9*, respectively.

we have shown that Mac-2 is an inducible macrophage antigen, because its expression and synthesis by peritoneal macrophages is highly dependent on the eliciting agent.

Two MAb—M3/38, an IgG2a, and M3/31, an IgM—define the Mac-2 antigen. Co-precipitation experiments showed that determinants recognized by both antibodies are present on the same molecular species. Therefore, M3/38 HL2, a M3/38 variant secreting only the specific heavy and light chains, was employed in most experiments described herein.

Mac-2 is expressed on the surface of TG-elicited macrophages as shown by immunofluorescence, direct binding, and ^{125}I-labeling, and is synthesized by these cells as shown by ^{35}S-methionine incorporation. Mac-2 is a molecule of 32,000 M_r as shown by immunoprecipitation and SDS-PAGE. The identical mobility of Mac-2 in SDS-PAGE under reducing and nonreducing conditions indicates the absence of interchain disulfide bonds. Sometimes, fourfold to 25-fold lower amounts of a 30,000 M_r polypeptide were also seen on SDS-PAGE both after ^{125}I and [^{35}S]-methionine labeling. It seems likely that the 30,000 M_r chain is a degradation product of the 32,000 M_r chain, because it was not found in stoichiometric quantities, and because labeling with ^{125}I suggested it is not an intracellular precursor.

A 60,000 M_r polypeptide has also been found to be precipitated from ^{125}I-labeled PEC by M3/31 and M3/38. The amount in some preparations (not shown here) was similar to that of the 32,000 M_r polypeptide. Precipitation was specific, because it did not occur with normal IgG or Mac-1 or Mac-3 MAb (15). However, no trace of this 60,000 M_r polypeptide has ever been seen in over 10 different [^{35}S]-methionine-labeling experiments. Thus, the 60,000 M_r polypeptide appears to be an exogenously derived component that can become specifically associated with Mac-2 on the cell surface. The interaction may be that of a ligand with its receptor.

The mannose-fucose receptor of liver has an M_r of about 30,000 (23). Recent studies show that macrophages also bear this receptor (24). However, the M3/31 and M3/38 MAb to Mac-2 do not inhibit the mannose-fucose receptor (P. Stahl, personal communication), nor does yeast mannan inhibit the binding of M3/38 MAb to macrophages (unpublished observation). Furthermore, the receptor is found on both resident and TG-elicited macrophages, and thus has a different distribution than Mac-2 (24).

By saturation binding, TG-elicited macrophages have been found to bind 1.7 × 10^5 M3/38 MAb per cell. Depending on the proportion of M3/38 MAb bound bivalently, the number of Mac-2 cell surface molecules is between 1.7 × 10^5 to 3.4 × 10^5/cell. Mac-2 thus is a major surface component of TG-elicited macrophages and is present in sufficient quantity for chemical characterization.

Immunofluorescence flow cytometry studies showed that Mac-2 is expressed on <1% of cells from spleen, bone marrow, thymus, and lymph node. Immunoprecipitation studies also showed that Mac-2 is either absent from lymph node and spleen cells or present in less than 0.3% of the amount on

macrophages. Absorption studies showed that Mac-2 is abundantly expressed on TG-induced macrophages, that bone marrow and spleen have slight but significant absorbing capacity, and that lymph node and thymus are negative.

The finding that spleen and bone marrow are positive for Mac-2 by absorption but negative by the other techniques is probably because residual stroma were purposely not removed from tissues used in the absorption experiments. A high proportion of tissue macrophages remain adherent to stroma (25). We have also found that brightly Mac-1-positive splenic macrophages seen in thin sections do not appear to be released by teasing into single cell suspensions (26). Other tissues known to contain mononuclear phagocytes, such as lung and liver, also exhibit a low absorbing capacity.

The most striking finding of this study is that Mac-2 is preferentially associated with macrophages elicited by nonspecific inflammatory agents, especially TG. TG-elicited macrophages are >96% Mac-2 positive, whereas unelicited macrophages and macrophages elicited by *Listeria monocytogenes*, LPS, or Con A are <2% positive. The amounts of Mac-2 synthesized by TG-elicited macrophages, as determined by [^{35}S]-methionine incorporation, are 10 to 30 times higher than those synthesized by other macrophage subpopulations. This confirms that Mac-2 expression is highly induced in macrophages elicited by TG. Interestingly, the level of Mac-2 synthesis in macrophages recovered after treatment with TG for 1 day is 80% of that in macrophages recovered after 4 days. This suggests that the cells recruited into the peritoneum after 1 day are already committed to the synthesis of Mac-2. In parallel studies, we have also examined [^{35}S]-methionine incorporation into Mac-1 and Ia antigens by the same panel of macrophages as shown in Figure 9 (14). Macrophages elicited by different agents all synthesized similar amounts of Mac-1 antigen, in contrast to Mac-2. Interestingly, synthesis of Ia antigens also varied among the different macrophage preparations, but the pattern was distinct from that of Mac-2. Con A- and *Listeria*-elicited macrophages were highest and TG-induced macrophages lowest in incorporation of [^{35}S]-methionine into Ia. This correlates well with previous studies showing that Con A and *Listeria* selectively elicit macrophages with high levels of surface Ia expression (19).

Recently, several polypeptides have been identified by SDS-PAGE that also are preferentially associated with TG-elicited macrophages. Using lactoperoxidase-catalyzed surface iodination and SDS-PAGE, Yin *et al.* (27) found that inflammatory macrophages expressed high quantities of a 103,000 M_r glycoprotein. Kan-Mitchell and Mitchell (28) also compared iodinated polypeptides of macrophages elicited by TG, protease peptone, *C. parvum*, and bacillus Calmette-Guérin. They observed that three polypeptides of 64,000, 43,000, and 40,000 M_r were enriched only in TG-elicited cells. However, because studies with internal radioactive labeling or antibodies were not carried out, it is possible that these polypeptides are exogenously acquired or are degradation products of other surface moieties. Le Blanc *et al.* (29) developed a rat MAb to cultured bone marrow cells, termed 54-2. The antigen recognized by 54-2 is expressed on TG-elicited macrophages, cultured bone marrow macrophages, and mast cells, but not on resident macrophages, blood monocytes, granulocytes, alveolar macrophages, lymphocytes, or freshly explanted bone marrow cells. The polypeptide precipitated by 54-2 is 180,000 M_r by SDS-PAGE (our unpublished results) and thus resembles the antigen defined by M3/37 (15). Therefore, the 54-2 and Mac-2 antigens are distinct cell surface antigens.

Peritoneal exudate macrophages harvested after i.p. injection of inflammatory agents or bacteria are heterogeneous. In the light of the differences in biochemical, functional, and morphologic characteristics, macrophages elicited by diverse means can be viewed as distinct cell subpopulations. TG elicits higher numbers of peritoneal macrophages than other agents. TG-elicited macrophages are large, highly vacuolated, and highly phagocytic cells. They also exhibit elevated levels of plasminogen activator, elastase, and alkaline phosphodiesterase I (5). However, cell surface antigens preferentially induced in these cells had not previously been identified. It is not clear whether macrophages elicited by diverse agents are differentiating along distinct pathways or are induced to distinct stages of differentiation along the same pathway. Mac-2 is an intriguing marker for studies on these questions. As a major surface component that is specifically induced in macrophages responding to highly inflammatory stimuli, its further biochemical and functional characterization is also of considerable interest.

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