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Tissue Distribution, Structural Characterization, and Biosynthesis of Mac-3, a Macrophage Surface Glycoprotein Exhibiting Molecular Weight Heterogeneity*

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Mac-3 is a mouse macrophage differentiation antigen defined by a rat anti-mouse monoclonal antibody (MAb), M3/84. The structure, biosynthesis, quantitative surface expression, and distribution of Mac-3 have been studied by radiolabeling and isolation with MAb-Sepharose, saturation binding, absorption, and immunofluorescence flow cytometry. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Mac-3 migrates as a diffuse band with average M_r = 110,000. Labeling of intact cells with ^{125}I and accessibility to MAb show it is present at least in part on the cell surface. Saturation labeling with ^{125}I-MAb shows 4.2 × 10^4 cell surface sites on thioglycollate medium-elicited peritoneal macrophages. [^{35}S]Methionine and [^{3}H]glucosamine incorporation into Mac-3 by purified macrophages show it is a glycoprotein synthesized by these cells. Absorption shows Mac-3 is strongest in macrophages, present in lower quantities in lung, liver, bone marrow, and spleen, and undetectable in thymus, lymph node, brain, and heart. Immunofluorescent flow cytometry shows surface expression on thioglycollate-elicited macrophages but not bone marrow, spleen, lymph node, or thymus cell suspensions. Similar amounts of Mac-3 are immunoprecipitated from resident macrophages or macrophages elicited by sterile inflammatory agents, intracellular parasites, or immunomodulators, but the average M_r of Mac-3 varies from 92,000 to 110,000. Mac-3 is synthesized from precursor(s) of M_r = 74,000 and 79,000, identical in the different macrophages. Processing into the mature molecule, which migrates in sodium dodecyl sulfate-polyacrylamide gel electrophoresis as a more diffuse band and varies in M_r among macrophage elicited by different agents and to a lesser degree between different preparations of the same type of macrophage, occurs in 15 to 30 min.

Macrophages play an important role in phagocytosis of foreign organisms and materials, in antimicrobial immunity, in resistance to tumors and viruses, in induction and regulation of T lymphocyte immune responsiveness, in inflammatory responses, and in regulation of hematopoiesis (1-4). Macrophage cell surface proteins undoubtedly play an important role in many of these diverse processes. To provide a means for studying macrophage cell surface structures, anti-macrophage MAb[1] have been prepared (5, 6). MAb can facilitate the isolation of surface antigens for structural characterization, the study of macrophage differentiation and subsets, and the association of functions with particular cell surface structures.

We previously described the combination of MAb-immunoadsorbents and cell hybridization in a cascade procedure for the efficient construction of MAb libraries to macrophage surface antigens (7). Rats were immunized to a mouse macrophage glycoprotein fraction from which previously identified immunodominant antigens had been removed with MAb-immunoadsorbents, and their spleen cells were fused to mouse myeloma cells to obtain MAb-secreting hybridomas. One MAb which was obtained immunoprecipitated an antigen of M_r = 110,000 termed Mac-3. Here we describe the cell distribution and biochemical characterization of this antigen. It is found on macrophages and some nonlymphoid tissues, but not on lymphocytes. It is a glycoprotein showing a broad band in SDS-PAGE and is synthesized by macrophages. Since peritoneal resident macrophages and macrophage populations elicited by sterile inflammatory agents, intracellular facultative bacteria, and immunomodulators differ in enzyme levels and in functional capacities such as cytotoxicity, induction of immune responsiveness, and phagocytosis (8-12), they were also examined for differences in Mac-3 expression. The different populations all synthesized Mac-3, but the average M_r varied from 92,000 to 110,000. Biosynthesis experiments show that the Mac-3 precursor in all the populations is a doublet of M_r = 74,000 and 79,000. Thus, variation in post-translational processing of certain glycoproteins appears to be a previously undescribed source of macrophage diversity.

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies—M3/84.6.34, designated M3/84 for brevity, is a subcloned, stabilized rat spleen cell × NSI myeloma hybridoma obtained as previously described (7). The cell line has been deposited with the American Type Culture Collection (Rockville, MD), and purified M3/84 MAb can be obtained from Hybritech (San Diego, CA). M3/84 can be grown in Dulbecco's modified Eagle's or RPMI 1640 medium supplemented with horse or fetal calf serum, and for large scale MAb production was grown in spinner cultures supplemented with 5% fetal calf serum. M3/84 secretes an antibody containing specific rat γ1 and κ chains and no myeloma chains. The MAb was purified by $(NH_4)_2SO_4$ precipitation and DEAE and Sephadex G-

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[1] The abbreviations used are: MAb, monoclonal antibody(ies); Con A, concanavalin A; PAGE, polyacrylamide gel electrophoresis; PBS, 0.01 M $NaPO_4$, 0.14 M NaCl, pH 7.3; PEC, peritoneal exudate cells; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

200 chromatography as previously described (13).

Cell Preparation—Male C57/BL6 mice (Jackson Laboratory, Bar Harbor, ME), 8–16 weeks of age, were used in most experiments. Exudates were obtained after intraperitoneal injection of one of the following agents: 1.5 ml of Brewer's thioglycollate medium; 1.5 ml of 10% protease peptone medium; 40 μg of *Salmonella typhosa* lipopolysaccharide (Westphal) (all from Difco Laboratories, Detroit, MI); 15 μg of concanavalin A (Sigma); or 2×10^4 live *Listeria monocytogenes* organisms (the generous gift of Dr. Emil Unanue). The time of treatment was usually 4 days for thioglycollate and 3 days for the others. Peritoneal exudates or resident cells were harvested in PBS containing 20 units/ml heparin (Sigma).

Site Number Estimation—Purified M3/84 (100 μl, 0.1 mg/ml) was iodinated with 0.5–1 mol/mol of ^{125}I using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril as described (14), except that the reaction was terminated by transfer to fresh tubes containing 20 μl of 0.4 mg/ml L-tyrosine. Thioglycollate-elicited PEC (20 μl, 2.5×10^7/ml) were mixed with 80 μl of ^{125}I-M3/84 (specific activity, 3.6×10^6 cpm/μg) at varying concentrations. The amount of binding was determined after shaking 1 h at 4 °C as described previously (15).

Tissue Absorption Studies—Procedures for tissue absorption studies have been described in detail elsewhere (15). Briefly, limiting concentrations of ^{125}I-M3/84 MAb were absorbed with equal volumes of serially 5-fold diluted tissues in 96-well microtiter plates. Tissues had been homogenized in PBS. Cells were suspended in PBS. The protein concentration of tissues and cells was determined by the Lowry assay. Residual ^{125}I-M3/84 in supernatants ($2000 \times g$) was measured by binding to 10^6 thioglycollate-elicited PEC, and per cent binding was determined relative to mock absorption with PBS.

Immunofluorescent Flow Cytometry—Cells were incubated with MAb-containing spent culture supernatants, washed, labeled with affinity-purified fluorescein-isothiocyanate-rabbit F(ab')$_2$ anti-rat IgG, and analyzed on a Becton Dickinson FACS-II using glutaraldehyde-fixed sheep cells as standards as previously described (16).

Cell Labeling and Immunoprecipitation—Cells were surface labeled with ^{125}I by modification of the lactoperoxidase-glucose oxidase method (17). For biosynthetic labeling, 10^7 peritoneal exudate cells were plated on tissue culture flasks and incubated 18 h at 37 °C unless otherwise specified. Nonadherent cells were washed off and adherent cells were labeled with 200 μCi of L-[^{35}S]methionine (New England Nuclear) in methionine-free Dulbecco's modified Eagle's medium for 6 h or with 125 μCi of D-[1,6-^{3}H(N)]glucosamine hydrochloride (New England Nuclear) in Dulbecco's modified Eagle's medium containing 80 mg/liter (9% of normal) glucose for 1 h. All labeling media were supplemented with 10% dialyzed fetal calf serum. Cells were usually incubated with the appropriate labeling medium for 45 min at 37 °C before addition of radioisotopes. In some experiments, adherent cells were labeled in the presence of 5, 10, or 20 μg/ml of tunicamycin (generous gift of Eli Lilly Co., Indianapolis, IN) after a 2-h preincubation with the inhibitor. In pulse-chase experiments, adherent cells were labeled with [^{35}S]methionine as above. Cells were then incubated with warm Dulbecco's modified Eagle's medium supplemented with methionine and 10% fetal calf serum at 37 °C for varying periods either immediately after labeling or after two rapid washes with warm PBS. The adherent monolayer was then washed three times in PBS supplemented with 0.1 mM phenylmethylsulfonyl fluoride and detergent solubilized in 0.01 M Tris-HCl, pH 8.0, 0.14 M NaCl, 1% Triton X-100, 1% hemoglobin, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide. After centrifugation at 100,000 × *g* for 1 h, the supernatant was recovered. Lysates were diluted to about 200 μl with 0.01 M Tris-HCl, pH 8.0, 0.14 M NaCl, 0.1% Triton X-100, 0.1% hemoglobin, and shaken in 1.5-ml Microfuge tubes (Sarstedt) with 12 μg of M3/84 IgG or normal rat IgG coupled to 10 μl of Sepharose CL-4B for 90 min at 4 °C. Beads were washed twice with the same buffer, once with 0.01 M Tris-HCl, pH 8.0, 0.14 M NaCl, and once with 0.05 M Tris-HCl, pH 6.8. Laemmli sample buffer was added, the beads were heated in a 100 °C bath, and the supernatant was subjected to SDS-PAGE (18) and fluorography (19).

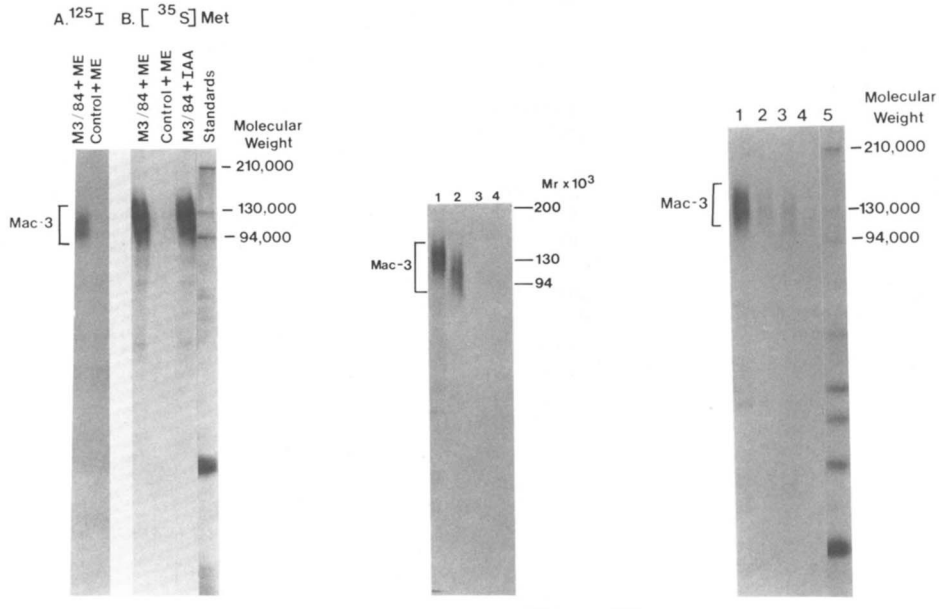

FIG. 1 (*left*). **SDS-PAGE of immunoprecipitates from ^{125}I- or [^{35}S]methionine-labeled macrophages.** Thioglycollate-elicited macrophage detergent lysates containing 3×10^5 cpm of ^{125}I (*A*) or 1.5×10^6 cpm of [^{35}S]methionine (*B*) were shaken with M3/84 or normal rat IgG coupled to Sepharose CL-4B, and the isolated material was treated with reduced sample buffer (+5% 2-mercaptoethanol, *ME*) or nonreduced sample buffer (+50 mM iodoacetamide, *IAA*) at 100 °C and subjected to SDS-10% PAGE and autoradiography with enhancing screens or fluorography, respectively. Molecular weight standards were myosin, β-galactosidase, and phosphorylase.

FIG. 2 (*center*). **SDS-PAGE of immunoprecipitates from thioglycollate-elicited macrophages labeled with [^{35}S]methionine or [^{3}H]glucosamine.** Detergent lysates containing 1.5×10^6 cpm of [^{35}S]methionine-labeled (*lanes 1* and *3*) or 3×10^5 cpm of [^{3}H]glucosamine-labeled cells (*lanes 2* and *4*) were shaken with M3/84 (*lanes 1* and *2*) or normal rat IgG (*lanes 3* and *4*) coupled to Sepharose CL-4B. Beads were washed, boiled in sample buffer containing 5% 2-mercaptoethanol, and eluates were subjected to SDS-PAGE and fluorography.

FIG. 3 (*right*). **Effect of tunicamycin on the immunoprecipitation of Mac-3 from [^{35}S]methionine-labeled macrophages.** Thioglycollate-elicited macrophages were labeled with [^{35}S]methionine in the absence or presence of tunicamycin, and equal quantities (1.5×10^6 cpm) of Triton X-100 lysates were subjected to isolation with M3/84-coupled Sepharose CL-4B and SDS-PAGE as described under "Experimental Procedures." The concentrations of tunicamycin used were as follows: *1*, none; *2*, 5 μg/ml; *3*, 10 μg/ml; and *4*, 20 μg/ml. Molecular weight standards (16) were run in *lane 5*.

RESULTS

Biochemical Characterization of Mac-3—When PEC were vectorially labeled with ^{125}I and the Mac-3 antigen was isolated from Triton X-100 lysates with M3/84 MAb coupled to Sepharose Cl-4B, a diffuse band in SDS-PAGE was consistently obtained (Fig. 1*A*). When macrophages were purified from PEC by adherence, and labeled with [^{35}S]methionine, the same band was isolated (Fig. 1*B*). The M_r of Mac-3 isolated from different batches of thioglycollate-elicited macrophages varies from 110,000 to 130,000 (Figs. 1–3). Mac-3 had the same mobility in SDS-PAGE whether reduced with 2-mercaptoethanol or treated with iodoacetamide (Fig. 1*B*). These results show that Mac-3 is present at least in part on the cell surface, is synthesized by macrophages, and does not contain interchain disulfide bonds.

Isolation of Mac-3 from [^{3}H]glucosamine-labeled macrophages showed that it contains carbohydrate (Fig. 2, *lane 2*). Mac-3 isolated from a different preparation of macrophages labeled with [^{35}S]methionine had a somewhat higher M_r (Fig. 2, *lane 1*). Variations from M_r = 130,000 to 110,000 are also seen among different batches of thioglycollate-elicited macrophages labeled with [^{35}S]methionine, and this therefore does not appear to be due to the type of label. As a further test for the glycoprotein nature of Mac-3, the effect of tunicamycin on its synthesis was studied. Tunicamycin blocks the formation of *N*-acetylglucosaminyl pyrophosphoryldolichol, thereby inhibiting *N*-linked glycosylation (reviewed in Ref. 20). Macrophages were pretreated with tunicamycin and then labeled with [^{35}S]methionine. Tunicamycin inhibited Mac-3 biosynthesis partially at 5 and 10 μg/ml and completely at 20 μg/ml (Fig. 3, *lanes 2–4*). No lower M_r form of Mac-3 was detected. In parallel experiments, the same tunicamycin concentrations had no effect on Mac-2 biosynthesis (not shown). These experiments suggest that Mac-3 contains *N*-linked oligosaccharides. In the absence of *N*-glycosylation, it appears that Mac-3 is degraded or the antigenic determinant is lost.

Mac-3 was not labeled with [^{32}P]phosphate, despite labeling in the same preparation of the T200 or Ly-5 glycoprotein (results not shown), which has previously been shown to be phosphorylated (21).

Absorption of ^{125}I-M3/84 by Various Tissues—To study the expression of Mac-3 on different cells, quantitative tissue absorption studies were performed. Cell suspensions or tissue homogenates were incubated with ^{125}I-labeled purified M3/84 MAb. The amount of unbound antibody was assayed by binding to thioglycollate-elicited PEC. As shown in Fig. 4*A*, ^{125}I-M3/84 MAb was absorbed by PEC, to a lesser degree by spleen and bone marrow, but not by thymus or lymph node. By comparing the amount of protein required to achieve 50% absorption, spleen and bone marrow were found to absorb 10 and 4%, respectively, as much ^{125}I-M3/84 MAb as PEC.

Similar studies using nonlymphoid tissues showed that the absorbing capacities of liver and lung were 14 and 7% that of PEC (Fig. 4*B*). In contrast, little or no absorption was detected when heart or brain were used.

Immunofluorescent Flow Cytometry of Lymphoid Cells Labeled with M3/84 MAb—To further characterize the distribution of Mac-3, single cell suspensions were prepared from bone marrow, spleen, thymus, and thioglycollate-elicited peritoneal exudates. After staining by M3/84 MAb and fluorescein-conjugated F(ab')$_2$ rabbit anti-rat IgG, the cells were analyzed by flow cytometry. Macrophage-sized cells in thioglycollate-elicited peritoneal exudates were >86% stained by

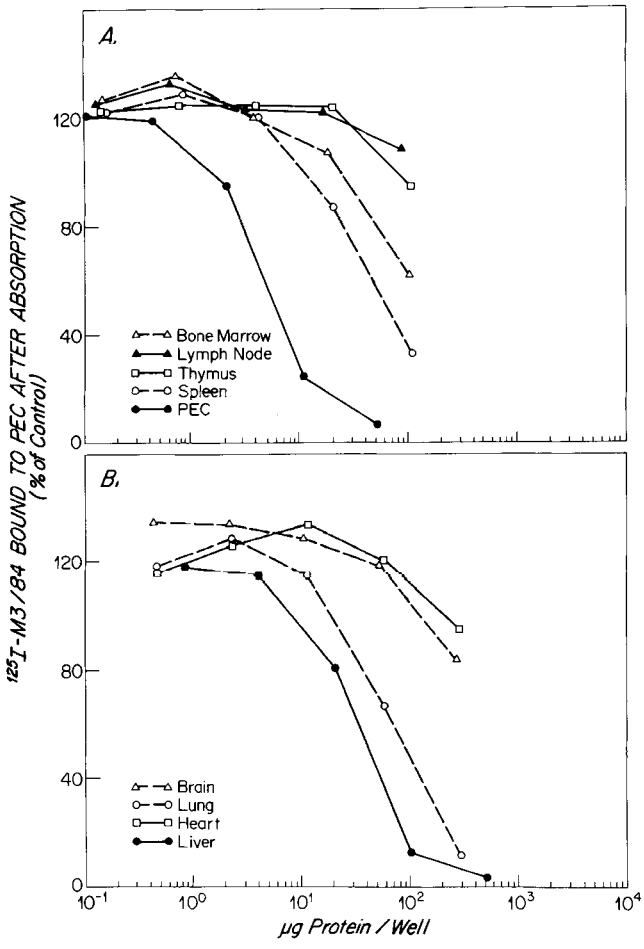

FIG. 4. **Absorption of ^{125}I-M3/84 with various mouse tissues.** Limiting amounts of ^{125}I-M3/84 were incubated with increasing quantities of lymphoid (*A*) or nonlymphoid (*B*) tissues. Residual binding to PEC was determined as described under "Experimental Procedures." Binding capacity was calculated as the percentage of ^{125}I-M3/84 bound after absorption with PBS.

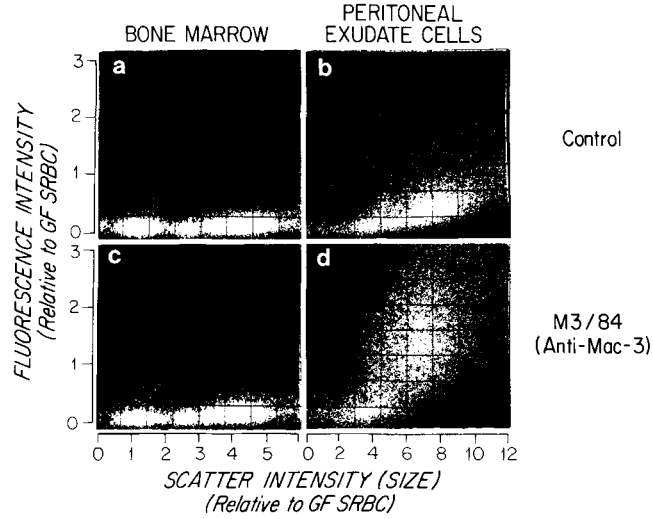

FIG. 5. **Immunofluorescence flow cytometry dot plot analysis of thioglycollate-elicited peritoneal exudate cells and bone marrow cells.** *Dots* represent fluorescence and scatter intensities of individual cells. Cells were first labeled with M3/84 clonal supernatants or NSI supernatant containing 50 μg/ml normal rat IgG as control. The second stage reagent was affinity-purified fluorescein-isothiocyanate-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, polymorphs, and macrophages appear at scatter intensities of approximately 2–3, 3–5, and 6–10, respectively.

M3/84 whereas smaller cells, such as lymphocytes and granulocytes in these exudates, were not stained (Fig. 5*d*). Essentially all (>98%) cells in single cell suspensions from bone marrow (Fig. 5*c*), spleen and thymus (not shown) were also found to be Mac-3$^-$ by immunofluorescence flow cytometry.

Estimation of M3/84 MAb-binding Sites on Thioglycollate-elicited Macrophages—To estimate the number of Mac-3 molecules on the surface of thioglycollate-elicited macrophages, the number of M3/84 MAb-binding sites was measured. Binding of ^{125}I-M3/84 MAb reached saturation above 20 nM (Fig. 6). Addition of excess unlabeled M3/84 showed that nonspecific binding was less than 5%. In three different experiments, the number of binding sites/thioglycollate-elicited macrophage was found to be $4.2 \pm 0.3 \times 10^4$. It is not clear what percentage of antibody was bound bivalently. Depending on the degree of bivalent binding, the number of Mac-3 sites/cell could range from 4.2 to 8.4×10^4.

Immunoprecipitation of Mac-3 from Peritoneal Macrophages Elicited by Different Agents and from Spleen and Lymph Node Cells—Previous studies showed that peritoneal macrophages elicited by different agents differ in their expression of Mac-2 (22) and Ia antigens (15, 23), even though they all bear Mac-1 (15). To explore the distribution of Mac-3 among macrophage populations, peritoneal exudates were elicited by thioglycollate, peptone, Con A, lipopolysaccharide, or *L. monocytogenes*. Exudate or resident macrophages were purified by adherence to tissue culture flasks and labeled with [^{35}S]methionine. For comparison purposes, spleen and lymph node cell suspensions were also labeled. Triton X-100 supernatants (100,000 × *g*) were dialyzed, and aliquots containing equal quantities of incorporated radioactivity were used for Mac-3 isolation (Fig. 7). Similar amounts of Mac-3 were immunoprecipitated from unelicited macrophages and macrophages induced by the various agents. However, there was variation in the M_r of Mac-3 isolated from different macrophage populations. The average M_r of Mac-3 from thioglycollate-elicited macrophages was 110,000, whereas from unelicited macrophages it was 92,000. A trace of Mac-3 was detected in spleen cells but not in lymph node cell lysates.

Biosynthesis of Mac-3—To study Mac-3 biosynthesis, thioglycollate-elicited macrophages were pulsed with [^{35}S]methionine and then chased in complete medium for varying lengths

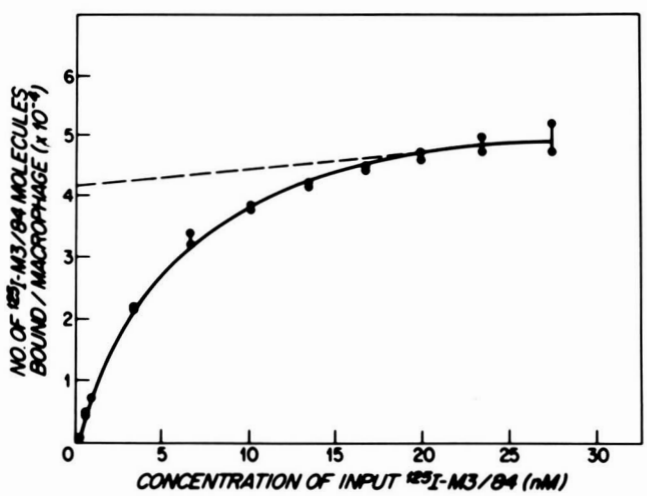

FIG. 6. **Saturation binding of ^{125}I-M3/84 to thioglycollate-elicited macrophages.** Increasing concentrations of ^{125}I-M3/84 MAb were incubated with 3×10^5 4-day thioglycollate-elicited PEC and binding was determined as described under "Experimental Procedures." Differential counts of Wright-Giemsa-stained PEC showed that approximately 85% were macrophages. Specific activity of ^{125}I-M3/84 MAb was 3.6×10^6 cpm/μg.

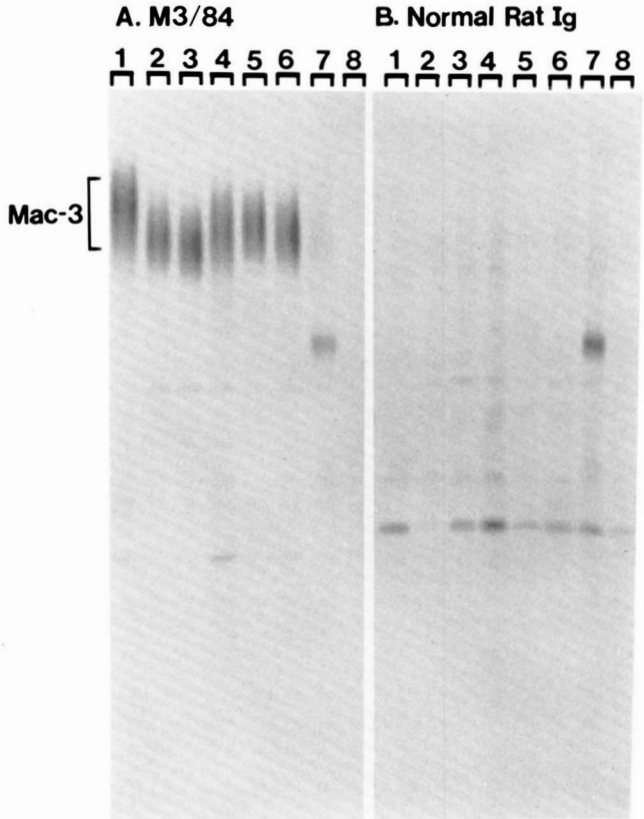

FIG. 7. **SDS-PAGE of [^{35}S]methionine-labeled Mac-3 antigen from macrophages elicited by various means and from spleen and lymph node cells.** Resident or exudate macrophages were purified by 2-h adherence on tissue culture flasks and labeled with [^{35}S]methionine. Single cell suspensions from spleen 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 12 μg of M3/84 coupled to Sepharose CL-4B. Controls were shaken with 12 μg of normal rat IgG coupled to Sepharose CL-4B. Beads were washed and heated in SDS-sample buffer containing 5% 2-mercaptoethanol, and the eluates were subjected to SDS-10% PAGE and fluorography. Macrophages were elicited with the following agents. *Lane 1*, thioglycollate; *2*, none (unelicited); *3*, Con A; *4*, lipopolysaccharide; *5*, *L. monocytogenes*; *6*, protease peptone. Spleen and lymph node cells were used for *lanes 7* and *8*, respectively.

of time. No antigens of $M_r = 110{,}000$ could be detected in cells labeled for 0.25 h (Fig. 8). Instead, two polypeptides of $M_r = 74{,}000$ and 70,000 were seen. Increasing the pulse labeling time to 0.5 h resulted in precipitation of small amounts of the $M_r = 110{,}000$ as well as the $M_r = 74{,}000$ and 79,000 bands. After chasing for 0.5 h or longer, the two lower M_r bands disappeared. Concomitantly, the intensity of the $M_r = 110{,}000$ band increased. Therefore, the mature Mac-3 antigen of $M_r \sim 110{,}000$ is derived from two precursor forms of $M_r = 74{,}000$ and 79,000. Moreover, processing of the precursors to form the mature antigen occurs within 15 to 30 min. By comparing autoradiograms exposed for different periods of time, the intensities of the Mac-3 polypeptides from cells chased for 22 h were found to be ⅕ that from cells chased for 3 h. This suggests that the half-life of Mac-3 is approximately 8 h.

The variation in the M_r of Mac-3 synthesized by macrophages elicited by different agents could be due to differences in precursor processing. To elucidate this point, unelicited macrophages as well as macrophages elicited by thioglycollate, Con A, lipopolysaccharide, and peptone were pulse-chase labeled with [^{35}S]methionine. After pulse labeling for 0.25 h, the $M_r = 74{,}000$ and 79,000 polypeptides were seen in lysates of all four macrophage subpopulations (Fig. 9*A*). After chasing

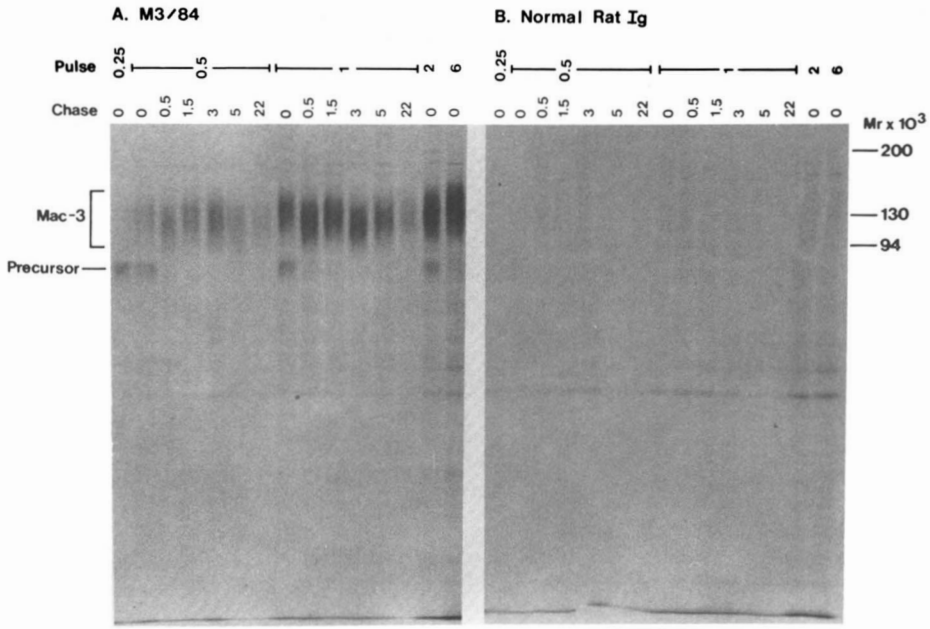

FIG. 8. **Pulse-chase labeling of Mac-3.** Adherent macrophages obtained after overnight culture were pulse labeled with [^{35}S]methionine and chased for the times indicated (expressed in hours). Cell lysates (1×10^6 cell equivalents) were immunoprecipitated with (*A*) 12 μg of M3/84 or (*B*) normal rat IgG coupled to Sepharose CL-4B and subjected to SDS-10% PAGE and fluorography.

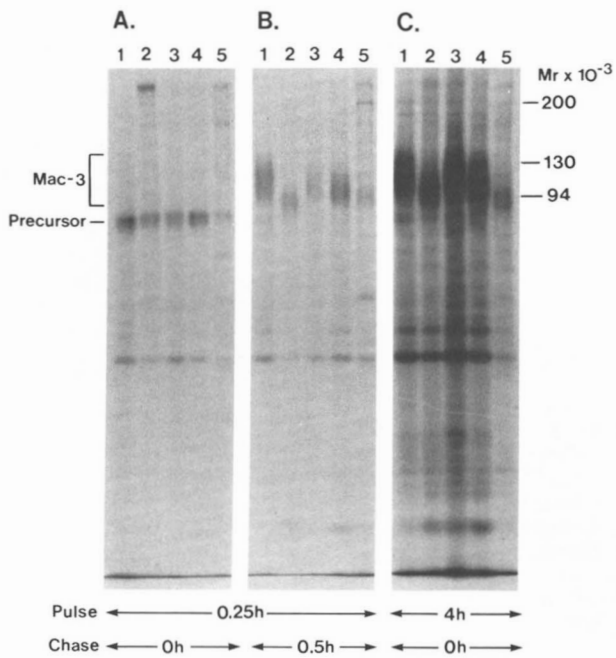

FIG. 9. **Biosynthesis of Mac-3 by macrophages elicited by different agents.** Peritoneal exudate cells elicited by different agents and resident peritoneal cells were incubated in tissue culture flasks for 3 h at 37 °C. Nonadherent cells were washed off and adherent cells were labeled with [^{35}S]methionine and chased for the indicated lengths of time. Cell lysates (2.7×10^5 cell equivalents) were immunoprecipitated with 12 μg of M3/84 coupled to Sepharose CL-4B and eluates were analyzed by SDS-PAGE and fluorography. Lysates used were from macrophages elicited by the following: *lane 1*, thioglycollate; *lane 2*, concanavalin A; *lane 3*, lipopolysaccharide; *lane 4*, 10% protease peptone; *lane 5*, none. No Mac-3 bands were seen when lysates were immunoprecipitated with normal rat IgG coupled to Sepharose CL-4B (not shown).

for 0.5 h (Fig. 9*B*), the mature but not the precursor form of Mac-3 was detected. The M_r of Mac-3 from different cells was different, with the average M_r ranging from 95,000 (for unelicited macrophages) to 107,000 (for thioglycollate-elicited cells). Heterogeneity was also observed when the chase period was extended to 90 min (not shown) or when cells were pulsed for 4 h (Fig. 9*C*). The average M_r of Mac-3 isolated from thioglycollate-elicited macrophages pulsed for 4 h remained the highest (112,000) and that from resident macrophages the lowest (92,000).

DISCUSSION

In a previous report, we described the use of cell hybridization and MAb immunoabsorbents in a cascade series for the efficient construction of MAb libraries to complex biological systems (7). The M3/84 MAb was obtained after immunization of rats with *Lens culinaris* lectin-purified mouse macrophage glycoproteins which had been depleted of several previously defined antigens with MAb immunoabsorbents. Here, we have defined the structure, cell distribution, and biosynthesis of the Mac-3 mouse macrophage differentiation antigen.

Mac-3 on intact cells is accessible to antibody and to vectorial iodination, showing that it is present at least in part on the cell surface. Immunoperoxidase staining of thin sections has shown that Mac-3 is also present in cytoplasmic granules (24).[2] Labeling of purified cells with [^{35}S]methionine and [^{3}H]glucosamine showed that Mac-3 is a glycoprotein and is synthesized by macrophages. Tunicamycin inhibited the appearance of Mac-3. Mac-3 has an average M_r = 110,000 in SDS-PAGE under both reducing and nonreducing conditions. It could not be labeled with [^{32}P]phosphate. Saturation binding studies showed 4.2×10^4 sites/cell.

The distribution of Mac-3 was studied by immunofluorescence, [^{35}S]methionine labeling, and absorption. By surface immunofluorescence, Mac-3 was found on macrophages but not on lymphocytes, thymocytes, erythrocytes, or bone marrow cells. This agrees with a study of immunoperoxidase staining of thin sections, except that Mac-3 was also found located intracellularly in 50% of bone marrow cells by the latter technique (24). By [^{35}S]methionine labeling, Mac-3 was readily detected in macrophages and in trace amounts in spleen, but not in lymph node.

Of a number of tissue and cell suspensions examined, macrophages gave the strongest absorption on a protein weight basis. Spleen and bone marrow gave weaker absorption while absorption by lymph node, thymus, brain, and heart was not detected. Liver and lung gave 14 and 7% as much absorption

[2] Flotte, T. J., Springer, T. A., and Thorbecke, G. J. (1982) *Am. J. Pathol.*, in press.

as macrophages, quantities suggesting that cells other than macrophages in these tissues might be Mac-3$^+$. Indeed, immunoperoxidase staining (24)[2] has shown that liver Kupffer and parenchymal cells are Mac-3$^+$, and that bile canaliculi are particularly strongly stained. In lung, alveolar macrophages and bronchial columnar epithelial cells are Mac-3$^+$. These studies have shown that Mac-3 is present on epithelial and endothelial cells and macrophages in a number of tissues and have confirmed the absence of Mac-3 from lymphocytes. In many of the cells which are positive by immunoperoxidase, Mac-3 is found in cytoplasmic granules. In intestinal columnar epithelial cells, the granules are localized to the apical portion of the cells. The finding of Mac-3 both in cytoplasmic granules and at the cell surface raises the possibility that it may cycle from one location to the other.

By [^{35}S]methionine labeling, we showed that Mac-3 was present in resident peritoneal macrophages and peritoneal macrophages elicited by thioglycollate, Con A, lipopolysaccharide, and peptone. These macrophage populations differ in functional and enzymatic activities (8–12). Together with the immunofluorescent studies and the findings that Mac-3 is present on macrophages in diverse anatomic sites (24),[2] this suggests that Mac-3 is a general marker for macrophages and can be used to distinguish these cells from lymphocytes.

Its $M_r \sim$ 110,000 and its cell distribution differentiate Mac-3 from previously characterized macrophage antigens, including Mac-1 (subunits of M_r = 170,000 and 95,000, Refs. 15, 25–27), Mac-2 (M_r = 32,000, Ref. 22), Ia (subunits of M_r = 34,000 to 25,000, Ref. 28), the Fc receptor (M_r = 60,000, Ref. 29), F4/80 antigen (M_r = 160,000, Ref. 30), 54-2 or Mac-4 antigen (M_r = 180,000, present on thioglycolate-elicited but not resident macrophages, Ref. 5, 31), and 2D2C antigen (M_r = 90,000, an alloantigen present on lymphocytes as well as macrophages, Ref. 32).

An unusual feature of the Mac-3 antigen is its M_r heterogeneity. In SDS-PAGE, the Mac-3 band is diffuse relative to other antigens such as Mac-1 and Mac-2 (*cf.* Refs. 15, 22). Among different preparations of thioglycollate-elicited PEC and within the same preparations at different time points in pulse-chase experiments (Fig. 8), there were slight variations in the average M_r of Mac-3. This was not due to an artifact of SDS-PAGE, because the mobility of a single Mac-3 preparation was the same in different electrophoresis experiments. More striking and consistent variations in M_r of Mac-3 antigen were found between peritoneal macrophages elicited by different agents. We reproducibly found that unelicited or Con A-elicited macrophage Mac-3 had the lowest M_r = 92,000 to 95,000, while thioglycollate-elicited macrophage Mac-3 had the highest M_r = 107,000 to 130,000. Even more striking differences have been found for macrophage cell lines, which have Mac-3 varying in M_r from 95,000 to 170,000.[2] Resident macrophages and macrophages elicited by sterile inflammatory agents, facultative intracellular parasites, and immunomodulators differ in tumoricidal and microbicidal activity, phagocytosis, spreading activity, receptor and enzyme activities, and cell surface antigen expression (5, 8–12). Similar differences are found among macrophage tumor cell lines (33).[3] Variation in M_r, and hence chemical structure, of certain cell surface glycoproteins such as Mac-3 is a previously unappreciated means for the generation of diversity among macrophages in different stages of differentiation. It is tempting to speculate that the heterogeneity of Mac-3 contributes to the specificity of the interactions between macrophages and other cells.

[3] Ralph, P., Ho, M.-K., Litcofsky, P. B., and Springer, T. A. (1982) *J. Immunol.*, in press.

Several cases of M_r variation have been described for lymphocyte antigens. The Thy-1 antigen isolated from brain and thymus has an M_r = 17,800 and 18,700, respectively. The protein moiety has M_r = 12,500 and is probably identical in both forms, the differences being due to carbohydrate (34). Differences have been found in the M_r (35) and sialylation (36) of Thy-1 isolated from thymocytes, peripheral T lymphocytes, and T cell lines which correlate with T lymphocyte maturation. The Ly-5 antigen is M_r = 220,000 on B lymphocytes and 187,000 and 200,000 on T lymphocytes. Distinct biosynthetic precursors of the T and B lymphocyte forms of Ly-5 have been identified (37).

To determine whether the variation in Mac-3 M_r was due to the precursors or to processing, biosynthesis experiments were carried out. Five macrophage populations elicited by different agents which had mature Mac-3 of differing M_r values were all found to have the same biosynthetic precursors. The precursor(s) consist of two relatively sharp bands of M_r = 74,000 and 79,000 which are converted in 15 to 30 min to the mature form. The heterogeneity in Mac-3 M_r is thus due to processing, which is most likely attributable to glycosylation. If so, this would suggest that Mac-3 contains a substantial amount of carbohydrate. The increase in M_r from the average of the precursors, 76,000, to 110,000 is 45% and the precursors would be expected to already bear high mannose glycans (20).

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