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Macrophage Differentiation Antigens: Markers of Macrophage Subpopulations and Tissue Localization

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Using the Kohler-Milstein myeloma-hybrid technique, (5) our laboratory has developed monoclonal antibodies recognizing four distinct antigens which are present on macrophages, but not lymphocytes. This demonstrates the distinctiveness of macrophage cell surface architecture (Table I). In this paper, we shall review the characteristics of these antigens. Two applications of anti-macrophage monoclonal reagents are also described, namely, their use in phenotyping macrophage subpopulations and in identification of macrophages in tissue sections.

MAC-1 ANTIGEN

M1/70 antibody, identifying the Mac-1 antigen, was obtained by a serendipitous route. Rats were immunized with mouse spleen cells and 10 cloned hybridoma lines were obtained (9). The M1/70 line gave barely significant, 2-fold over background binding to spleen cells in the indirect ^{125}I-anti-rat IgG sandwich assay. Screening on a tumor cell panel revealed that the P388D1 macrophage-like cell line bound 100-fold more M1/70 antibody than spleen cells, but a series of B and T lymphoid lines gave no significant binding. This led to further studies on the cell distribution and molecular properties of the antigen defined by M1/70, which has been designated Mac-1 (10). In normal tissues M1/70 gave binding to the adherent and nonadherent fraction of peritoneal exudate cells that was proportional to the number of macrophages in each. Immunofluorescent flow cytometry analysis showed that Mac-1 is expressed on thioglycollate-induced peritoneal exudate macrophages, 50% of bone marrow cells (Fig. 1), granulocytes, blood monocytes, and a small proportion of spleen cells but is absent from thymocytes and lymph node cells (Table 1). Peritoneal exudate macrophages express about 10-fold greater amounts of Mac-1 than positive cells in bone marrow (Fig. 1g,h), about 8-fold more than M1/70$^+$ spleen cells, and much more than granulocytes or blood monocytes (10). The increase in Mac-1 expression during maturation of monocytes to exudate macrophages is paralleled by a decrease in another antigen, the M1/69 heat stable antigen (HSA) (10). Mac-1 contains 2 polypeptide chains of 170,000 and 95,000 Mr which are

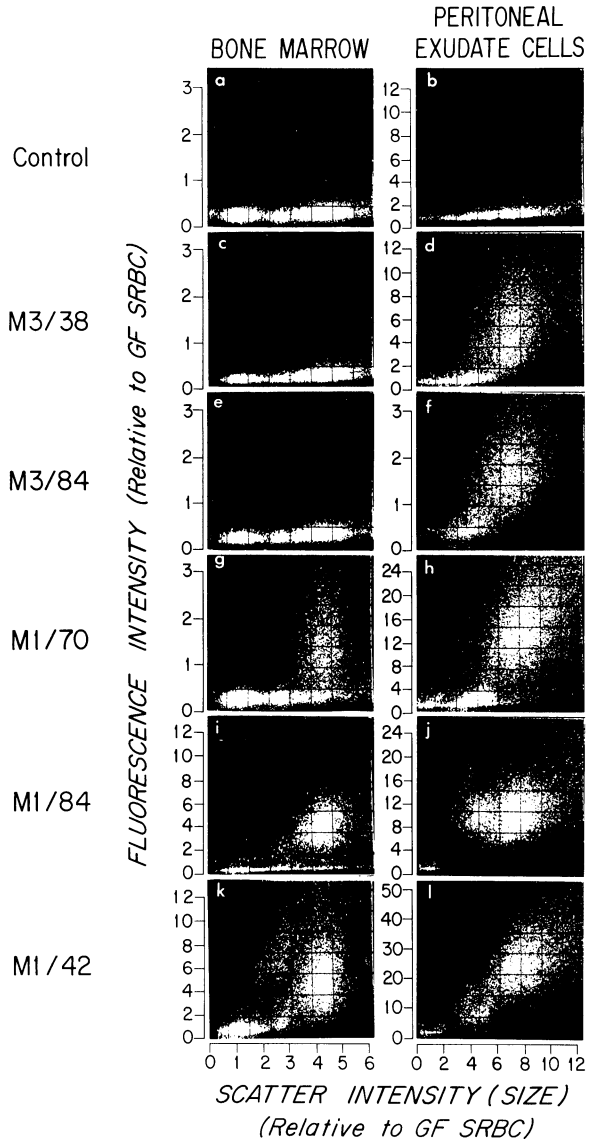

FIG. 1. FACS dot plot analysis of staining of Mac-2 (M3/38), Mac-3 (M3/84), Mac-1 (M1/70), M1/84, and H-2 (M1/42) on B6/J bone marrow and thioglycollate-induced 4 d PEC. Cells were labeled with rat MAb, then FITC-rabbit $F(ab')_2$ anti-rat IgG absorbed with mouse IgG as described previously (10). Mouse RBC, polymorphs, and macrophages appear at scatter intensities of 1, 4-5, and 7-10, respectively. GF SRBC = glutaraldehyde-fixed sheep red blood cells.

TABLE 1. Monoclonal rat anti-mouse macrophage antibodies

Antibody	Antigen	Polypeptide chains	Distribution* Positive	Negative	Antibody bound/TG-PEM	Reference
M1/70	Mac-1	170,000 95,000	(PP, LM, LPS, Con A TG)-PEM, PM, SM, M, G, 50% BM, NK	T, PLN	1.8×10^5	(2,10) Footnote 2
M3/31 M3/38	Mac-2	32,000	TG-PEM 20% PP-PEM†	>97% (LM, LPS, Con A)-PEM, PM, SM, G, T, PLN, >99% BM	1.7×10^5	(12) Footnote 2
M3/84	Mac-3	110,000	(PP, LM, LPS, Con A, TG)-PEM, PM	T, PLN, >99% BM	3.6×10^4	(11,12)
M3/37**	Mac-4	180,000	TG-PEM	T, >99% BM	ND	(11,12)

*Abbreviations used, PEM: peritoneal exudate macrophages, PM: unelicited peritoneal macrophages, SM: splenic macrophages, M: monocytes, G: granulocytes, BM: bone marrow cells, T: thymus cells, PLN: peripheral lymph node cells, TG: thioglycollate, PP: protease peptone, LM: L. monocytogenes, LPS: lipopolysaccharide, Con A: concanavalin A, NK: natural killer cells, ND: not done.

**not cloned.

†weakly positive.

precipitated from both ^{125}I-surface labeled and [^{35}S]-methionine labeled PEC. Mac-1 is expressed on all types of macrophages thus far examined. Both resident peritoneal macrophages and peritoneal macrophages elicited by sterile inflammatory agents, bacteria, and immunomodulators biosynthesize (Fig. 5A) and surface express (Fig. 4) Mac-1 antigen.

M1/70 also cross-reacts with an antigen on human monocytes, granulocytes, and natural killer (NK) and antibody-dependent cytotoxic (ADCC) cells (2). Presence on the latter cells was determined by fluorescence-activated cell sorting of monocyte-depleted blood mononuclear cells using F(ab')$_2$ M1/70 and fluorescein isothiocyanate (FITC)-labeled second antibody followed by testing for functional activity. M1/70$^+$ cells were enriched for NK and ADCC activity, while M1/70$^-$ cells were depleted of such activity when compared to stained but unsorted cells. Similar studies in mice reveal that NK and ADCC activity in mouse spleen or peritoneal exudates after infection with Listeria monocytogenes is also associated with M1/70$^+$ cells[1].

IMMUNOADSORBENT-CELL HYBRIDIZATION CASCADES

To obtain lineage-specific MAb, animals are usually immunized with whole cells of one lineage and MAb recognizing widely-shared antigens are screened out by testing on other types of cells. A major problem with this approach is that widely-shared antigens as well as the more readily identified macrophage-specific antigens may be immunodominant and thus may nonspecifically suppress the response to other antigens (8). In the M1 experiment, for example, the widely shared HSA and common leukocyte antigen (CLA) appeared immunodominant, since they accounted for 7/10 of the MAb obtained (9). These two antigens are also on peritoneal exudate cells. Immunization with whole PEC resulted in comparable titers of antibodies to Mac-1, HSA, and CLA as determined by competitive inhibition of the binding of [^{3}H]-labeled MAb to target cells (12). Therefore, in order to increase the chance of obtaining macrophage-specific MAb, these 2 antigens were removed by an immunoadsorbent procedure (Fig. 2). As illustrated, one of the attractive features of this method is that it may be indefinitely extended in a cascade so that the immunogenic stimulus is always limited to those antigens not yet identified by MAb.

MAC-2, 3 AND 4 ANTIGENS

To obtain further anti-macrophage MAb, rats were immunized with immunoadsorbent-depleted purified macrophage glycoproteins and their spleen cells fused with NSI as described in the legend of Figure 2. Hybrid culture supernatants were screened for binding to macrophages, and all binders were then screened for immunoprecipitation of ^{125}I-labeled cell surface antigens (12). Five supernatants precipitated polypeptides of 170,000 and 95,000 Mr which resemble those of Mac-1. Eleven cultures precipitated a 32,000 Mr polypeptide, one a 110,000 Mr,

[1]Holmerg, L.A., Springer, T.A., and Ault, K.A. (1981): Natural killer activity in the peritoneal exudates of mice infected with Listeria monocytogenes: characterization of the natural killer cells using a monoclonal rat anti-murine macrophage antibody (M1/70). Manuscript submitted.

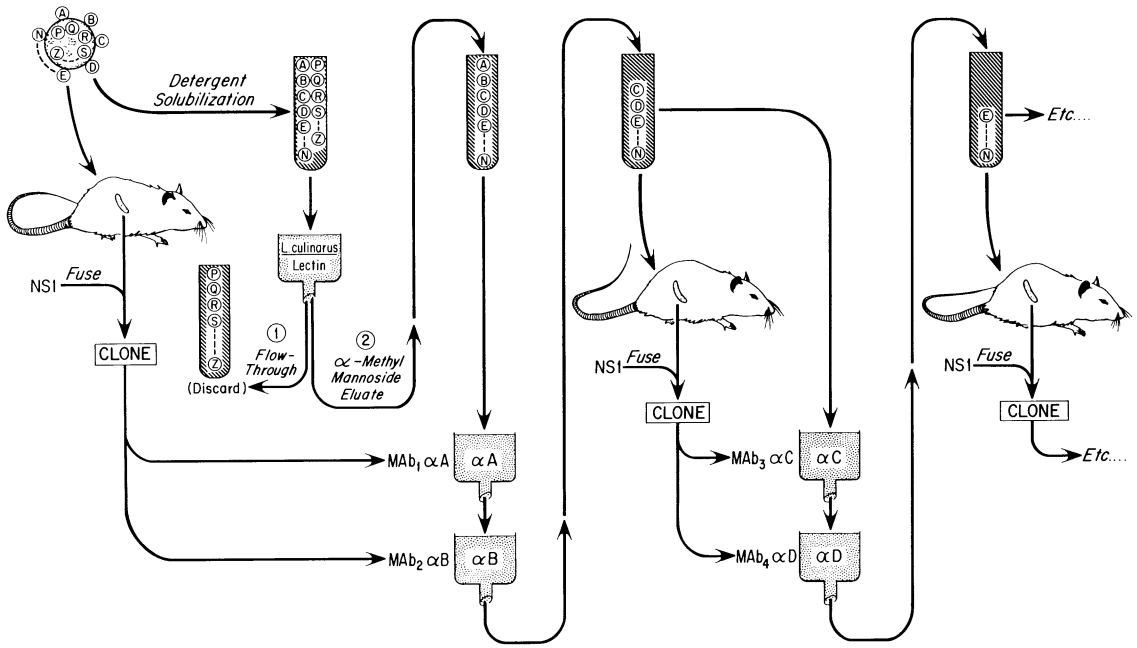

FIG. 2. Cascade immunoadsorbent procedure for focusing hybridoma production on unrecognized antigens. B6/J PEC (8×10^8 4d thioglycollate-induced, 84% macrophages, 2.5% neutrophils, 7.5% eosinophils, 4.5% lymphocytes, 1.5% RBC) in 20 ml PBS + 0.5 mM phenylmethylsulfonyl fluoride were N_2 cavitated after 10 m at 600 psi. Membranes were prepared, solubilized with sodium deoxycholate (NaDC) and glycoproteins purified on L. culinaris lectin columns. Samples were then passed through columns containing approximately 1 ml of Sepharose CL-4B coupled with 1 mg of purified MAb M1/69.16 (to remove HSA) and M1/89.23 (to remove CLA). The sample was dialyzed 7 days versus 2 changes daily of 0.01 M tris HCl, pH 8.2, to remove NaDC. Rats were injected twice with 40 µg protein of antigen in CFA and 4 months later with 100 µg in saline, and spleen cells fused 3 days later with NSI and grown in 5 x 96 well 0.2 ml culture trays. Full details have been described elsewhere (12).

and another a 180,000 Mr polypeptide. However, none of the cloned lines precipitated the 200,000 Mr CLA polypeptide (11). Analysis of the sera from animals immunized with cascade-purified antigen also showed absence of CLA competing or precipitating antibodies, although these antibodies were elicited by whole macrophages (12).

Three stable subcloned lines were obtained by the cascade procedure. (For brevity, subclone designations will be omitted.) A 32,000 Mr polypeptide (Mac-2), is precipitated by M3/31 and M3/38, while a 110,000 Mr polypeptide (Mac-3) is precipitated by M3/84 (Fig. 3A). A 180,000 Mr polypeptide (Mac-4) is precipitated by an uncloned line, M3/37 (Fig. 3B). (A cloned line, 54-2 has been independently obtained which appears to recognize the same antigen as determined by comigration in SDS-PAGE) (6). Side-by-side comparison of the high Mr Mac-4 (M3/37), Mac-3 (M3/84) and Mac-1 (M1/70) chains shows them all to have different Mr's and thus, they are all clearly unique antigens (Fig. 3B).

Mac-2 and 3 are present on essentially all thioglycollate-induced peritoneal exudate macrophages (Fig. 1) but on 0% of thymocytes, 0-1% of bone marrow cells and <10% of spleen cells (Table I). Mac-1 and Mac-2 are expressed in higher amounts on the surfaces of peritoneal macrophages than Mac-3 (Fig. 1), as is also evident from the numbers of M1/70, M3/38 and M3/84 bound per cell (Table I). All appear to be expressed in lower amounts on macrophages than H-2 antigen (Fig. 1, note the changes in fluorescence scale).

Mac-2 and 3 antigens are on the macrophage cell surface on the basis of fluorescent and ^{125}I labeling. ^{35}S-methionine incorporation into the polypeptides by the adherent fraction of thioglycollate-induced PEC also suggests these antigens are synthesized by macrophages (Fig. 3A).

Many preparations of Mac-2 antigen show a 30,000 Mr chain in addition to the 32,000 Mr chain. The quantity of this 30,000 Mr chain ranges from 1/4 to 1/24 that of the heavier chain, depending on the batch of cell lysate used. Much smaller amounts of slightly lower Mr polypeptides precipitated by M3/38 but not M3/31 have also been seen (Fig. 3A). The working hypothesis is that the smaller polypeptides are degradation products bearing the M3/38 but not the M3/31 determinant. Nevertheless, it is clear that determinants recognized by both MAb are on the same 32,000 Mr molecular species because preclearing of cell lysates by M3/31 depletes material precipitable by M3/38 and vice versa[2]. The M3/84 (Mac-3) 110,000 Mr band is characteristically 'fuzzy' after both ^{35}S-met and ^{125}I-labeling (Fig. 3a,b). This suggests a high degree of heterogeneity in the Mac-3 polypeptides. Indeed, Mac-3 antigens precipitated from different PEC subpopulations vary slightly in their Mr (Fig. 5).

The migration of Mac-1, Mac-2, and Mac-3 under reducing conditions (in the presence of 2-mercaptoethanol) is similar to that under non-reducing conditions (in the presence of iodoacetamide). Therefore, these antigens lack interchain disulfide bonds.

[2]Ho, M.K., and Springer, T.A. (1981): Mac-2, a novel 32,000 Mr macrophage subpopulation-specific antigen defined by monoclonal antibody. Manuscript submitted.

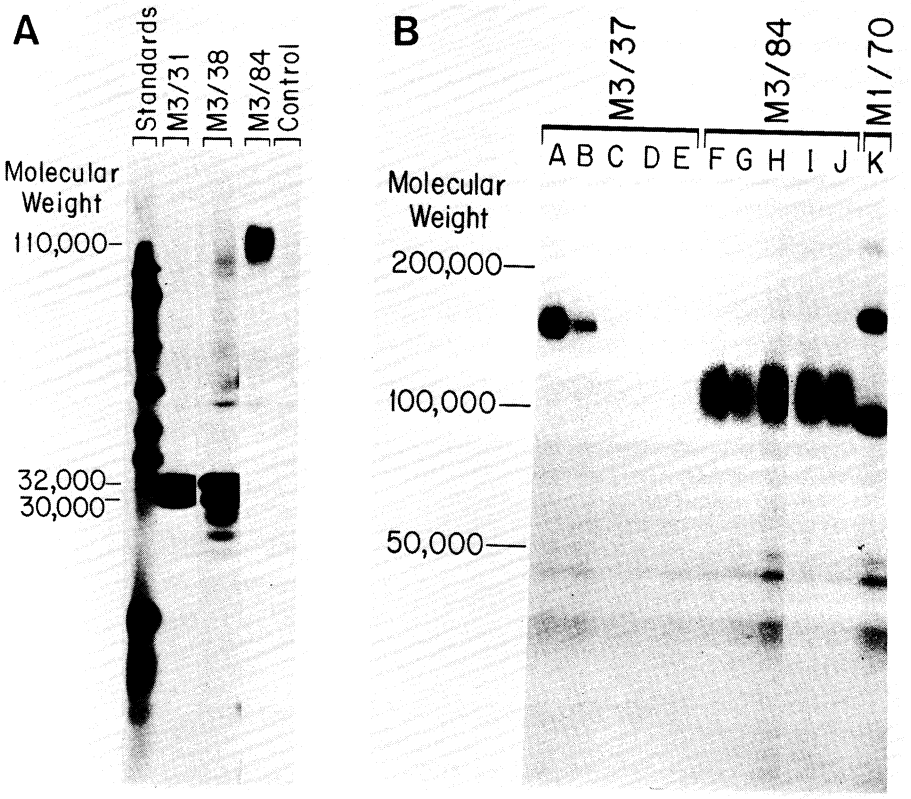

FIG. 3. SDS-PAGE of Mac-1, 2, 3 and 4 antigens immunoprecipitated from 4d thioglycollate-induced macrophages. Triton X-100 lysates of labeled cells were incubated with MAb, then with rabbit anti-rat IgG second antibody and precipitates analyzed by SDS-PAGE and autoradiography after 2-mercaptoethanol reduction (10). A. Cells adherent to tissue culture flasks were internally labeled with [^{35}S]-methionine. B. Cells were vectorially labeled with ^{125}I using lactoperoxidase. A-E: M3/37, uncloned supernatants at weekly intervals; F-H: M3/84 uncloned supernatants at weekly intervals; I: M3/84.6 clone; J: M3/84.6.34 subclone; K: M1/70.15.1 subclone.

The Mr and tissue distribution of these antigens differ from the macrophage Fc receptor II (13). Their relationship to the genetically defined Mph-1 antigen (1) is unknown.

MONOCLONAL ANTIBODIES AS MARKERS FOR MACROPHAGE SUBPOPULATIONS

Peritoneal macrophages elicited by various agents display different functions, ecto-enzymes, and morphology. Recently, we found that these macrophage subpopulations also express different macrophage differentiation antigen phenotypes. By immunofluorescent flow cytometry, Mac-1 is found on >86% of unelicited peritoneal macrophages, as well as macrophages elicited by thioglycollate, protease peptone, Con A, LPS and Listeria monocytogenes (Fig. 4). However, only thioglycollate-elicited macrophages are strongly stained for Mac-2. Peptone-elicited cells are 20% weakly positive whereas unelicited macrophages and those elicited by other agents are negative. SDS-PAGE of [^{35}S]-methionine-labeled Mac-2 shows that macrophages from thioglycollate-induced exudates synthesize 10-18x more Mac-2 than those from other exudates, and 30x more than unelicited macrophages (Fig. 5B). This correlates with the strong surface expression of Mac-2 on cells after thioglycollate treatment. In contrast, all macrophage subpopulations examined including unelicited macrophages, synthesize approximately the same amounts of both Mac-1 and Mac-3 (Fig. 5A,D). The average Mr of the Mac-3 polypeptides synthesized by different subpopulations ranged from 108,000 to 130,000. Together with the observation that the Mac-3 band is characteristically fuzzy, these results suggest that the Mac-3 antigens are heterogeneous, perhaps in glycosylation.

Previously, Beller and Unanue observed that the number of Ia$^+$ macrophages in peritoneal exudates elicited by Con A and live Listeria organisms are much higher than that in exudates elicited by thioglycollate, peptone or LPS (3). We obtained similar results by immunoprecipitating with M5/114, a rat anti-mouse Ia (unpublished) monoclonal antibody (Fig. 5E). Thioglycollate elicits peritoneal exudate macrophages with low Ia but high Mac-2 biosynthesis whereas the reverse is true for Con A and Listeria. Moreover, unelicited macrophages, and macrophages elicited by peptone and LPS have varying but low amounts of both antigens. Nevertheless, all these macrophages are positive for Mac-1 and Mac-3. Hence, in addition to functional and enzymatic differences, peritoneal macrophages elicited by various agents also display differing patterns of macrophage differentiation antigen expression.

IDENTIFICATION OF MACROPHAGES IN TISSUE SECTIONS BY INDIRECT IMMUNOFLUORESCENCE

The anatomical localization of lymphocyte subpopulations has been studied by histological, autoradiographic, and immunofluorescent methods (4,7,14,15). In contrast, little immunofluorescent staining of tissue macrophages has been performed due to the lack of specific antibodies to macrophage antigens. This prompted us to stain macrophages in frozen spleen sections with the M1/70 anti-Mac-1 MAb. We observed intense staining of cells with macrophage morphology in the red pulp (not shown) and marginal zone, but not in the T-dependent

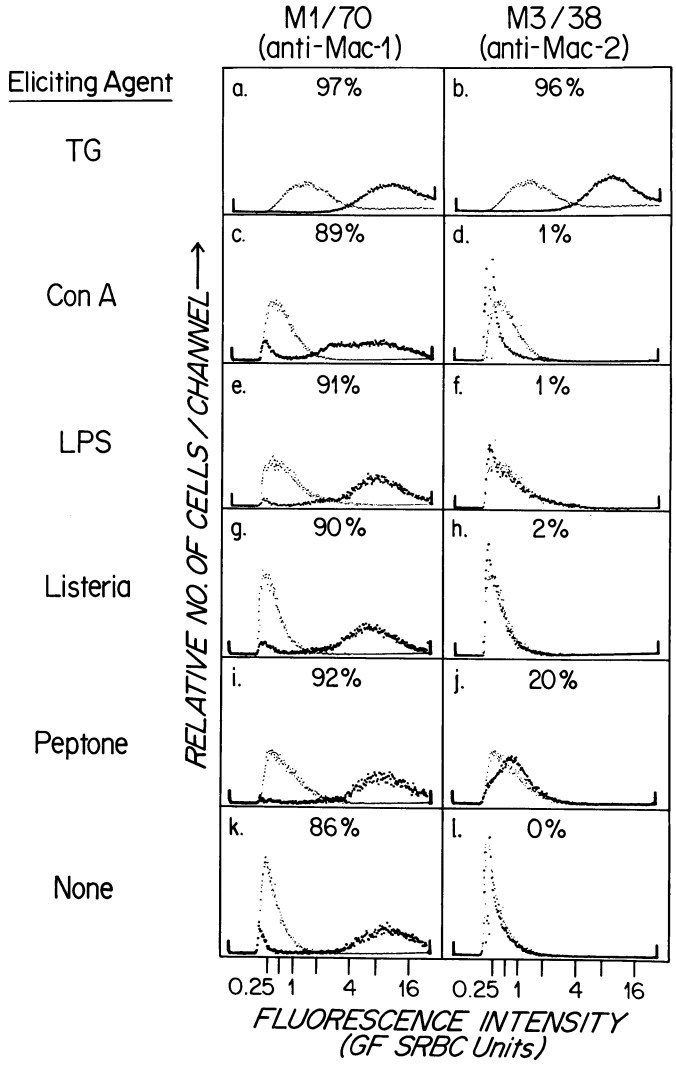

FIG. 4. Immunofluorescent flow cytometry analysis of Mac-1 and Mac-2 on peritoneal macrophages elicited by different means. Unelicited peritoneal macrophages were harvested by lavage from normal C57/BL6 mice whereas exudate macrophages were from mice injected intraperitoneally with one of the following: 1.5 ml Brewer's thioglycollate (TG), 15 μg concanavalin A (Con A), 40 μg Salmonella typhosa lipopolysaccharide (LPS), 1.5 ml 1% protease peptone, or 2 x 10^4 live Listeria monocytogenes organisms. The time of treatment was 4 days for TG and 3 days for the others. Cells were labeled with M1/70.15.1, M3/38.1.2 HL2, (bright curves) or NS-1 control supernatants plus 50 μg/ml normal rat IgG (dim curves) as described[2]. The scatter gates were set to exclude erythrocytes, dead cells, and most lymphocytes and polymorphs. The histograms shown are plotted with the relative cell number on a linear scale and fluorescence intensity on a logarithmic scale.

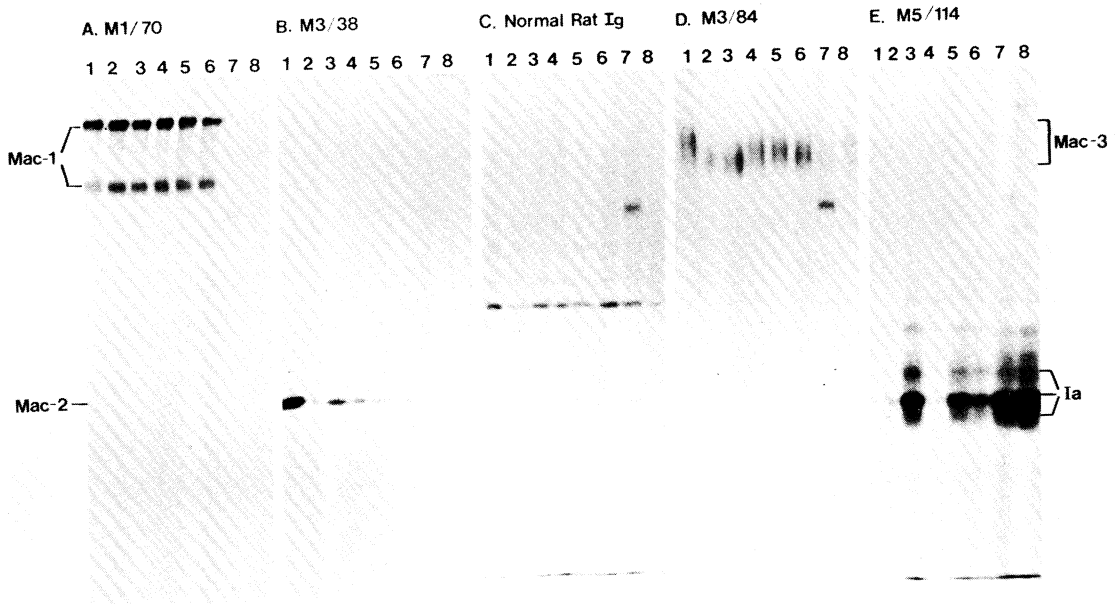

FIG. 5. SDS-PAGE of [^{35}S]-methionine-labeled Mac-1, Mac-2, Mac-3 and Ia antigens from macrophage elicited by various agents and from spleen and lymph node cells. Peritoneal macrophage subpopulations were obtained as in legend of Figure 4. They were purified by 2 h. adherence on tissue culture flasks, and labeled with 200 µCi L-[^{35}S]-methionine for 6 h. as described[2]. Spleen and lymph node cells were processed similarly except that the adherence step was omitted. Aliquots of Triton-X 100 cell lysates (1.5 x 10^6 cell associated cpm) were shaken with Sepharose CL-4B coupled with one of the following MAb: 8.7 µg M1/70.15.1, 8.5 µg M3/38.1.2 HL2, 12 µg M3/84.6.34, 20 µg M5/114, or 12 µg normal rat IgG for 90 min at 4°. Beads were washed, boiled in SDS-sample buffer containing 5% 2-mercaptoethanol, and the eluates analyzed on SDS-10% PAGE. Macrophage lysates used were elicited by one of the following agents: Lane 1, thioglycollate; Lane 2, unelicited; Lane 3, Concanavalin A; Lane 4, lipopolysaccharide; Lane 5, Listeria; Lane 6, peptone. Lanes 7 and 8 contain lysates of spleen and lymph nodes cells, respectively.

areas of white pulp (Fig. 6). Some granulocytes in the red pulp are also weakly stained due to their low expression of Mac-1. However, they can be easily differentiated from macrophages by their intensity of staining and morphology. This simple procedure can be used to study macrophages in other normal tissues, such as thymus and lung, or in pathological tissues, such as granulomas and tumors.

CONCLUSION

We have identified in these studies four macrophage antigens with distinct Mr and tissue distribution. Two of these, Mac-1 and Mac-3, are synthesized by all macrophage subpopulations examined thus far. However, Mac-2 seems to be preferentially associated with thioglycollate-elicited peritoneal macrophages. Ia antigens show a different pattern of expression. Therefore, macrophages can be defined into subsets with distinct antigenic phenotypes, as is the case for lymphocytes.

ACKNOWLEDGMENTS

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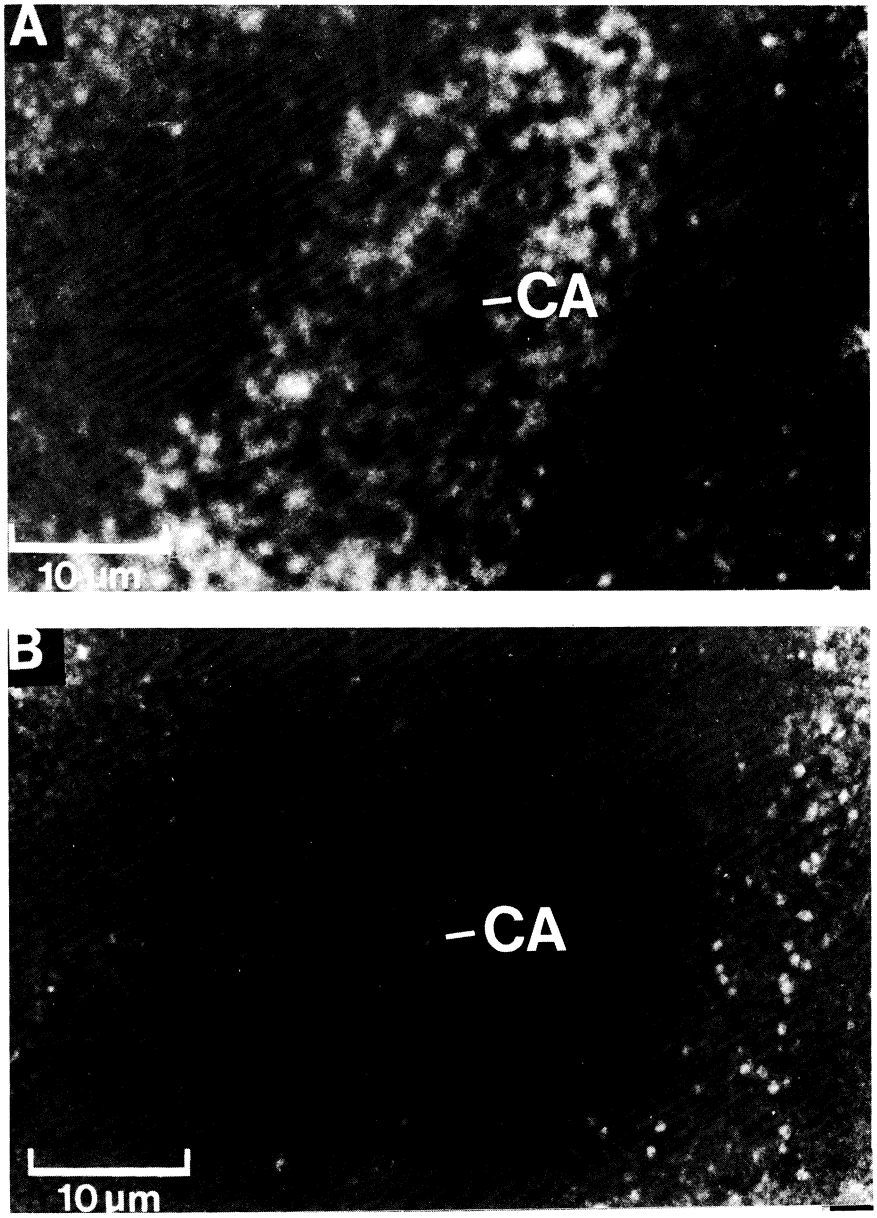

FIG. 6. Immunofluorescent staining of spleen sections with anti-Thy-1 and anti-Mac-1 MAb. Sections (3 µm) were cut from frozen BALB/c spleens, acetone fixed for 15', and washed in PBS (0.01 M phosphate, 0.14 M NaCl, pH 7.3). Adjacent sections were then stained with 40 µl of either (A) anti-Thy-1 (M5/49 MAb, 5 µg/ml) or (B) anti-Mac-1 (M1/70 MAb, 10 µg/ml) for 30 min. After two washes in PBS, they were further stained with 40 µl of FITC-rabbit $F(ab')_2$ anti-rat IgG for 30 min. All procedures were performed at room temperature. Thy-1^+ cells are located in the periarteriolar lymphatic sheath (A) whereas Mac-1^+ cells are scattered in the marginal zone (B). A branch of central artery (CA) can be seen in both sections.