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Mac-1,2,3 AND 4: MURINE MACROPHAGE DIFFERENTIATION ANTIGENS IDENTIFIED BY MONOCLONAL ANTIBODIES

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Recently, the Köhler-Milstein myeloma-hybrid technique has given great impetus to the analysis of cell surface complexity (Springer, 1980). Its most revolutionary feature is the ability to use highly complex antigens such as whole cells in xenogeneic immunization. The resultant multispecific response may then be resolved by cloning into a set of hybrid lines each secreting a monoclonal antibody (MAb) recognizing a single antigenic determinant on a single cell surface molecule. This paper will review some previously published as well as new work from this laboratory on the use of this technology for the identification and study of macrophage antigens.

Mac-1 ANTIGEN

M1/70 antibody, identifying the Mac-1 antigen, was obtained by a serendipitous route. Rats were immunized with mouse nylon-wool purified spleen cells and 10 cloned hybridoma lines were obtained (Springer et al., 1978). 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 P388D$_1$ 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 this antigen (Springer et al., 1979). In normal tissue M1/70 gave binding to the adherent and nonadherent fraction of peritoneal exudate cells that was proportional to the number of macrophages in each. FACS analysis showed that it is on peritoneal exudate macrophages, 50% of bone marrow cells (Fig. 1), and on granulocytes and blood monocytes, but found on only 4-8% of nylon wool purified

spleen cells and absent from thymocytes (Table 1) (Springer, 1980).

TABLE 1

MURINE MACROPHAGE DIFFERENTIATION ANTIGENS IDENTIFIED BY RAT MAb

Monoclonal antibody	Sub-class	Antigen polypeptide chain MW*	Designation	Tissue Distribution+ positive	negative
M1/70	γ2b	190,000; 105,000	Mac-1	PEM, M, G, 50% BM	T, R, >90% S
M3/31 M3/38	μ γ2a	32,000	Mac-2	PEM	T, R, >99% BM, >90% S
M3/84	γ1	110,000	Mac-3	PEM	T, R, >99% BM, >90% S
M3/37†	?	180,000	Mac-4	PEM	T, R, >99% BM, >90% S

* by SDS-PAGE after reduction

+ PEM, thioglycollate-induced 4d peritoneal exudate macrophages; M, blood monocytes; G, 1d exudate granulocytes; BM, bone marrow; T, thymus; R, red blood cells; S, spleen.

† not cloned

Mac-1 contains 2 polypeptide chains of 190,000 and 105,000 MW (Fig. 2B) and much greater quantities are precipitated from PEC than spleen. Peritoneal exudate macrophages express about 10-fold greater amounts of M1/70 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. The increase in M1/70 expression during maturation of monocytes to exudate macrophages is paralleled by a decrease in another antigen, the M1/69 heat stable antigen (HSA) (Springer et al., 1979). Because of its phagocyte-specificity and strong expression on macrophages, the M1/70 antigen has been designated Mac-1. M1/70 also cross-reacts with an antigen on human monocytes, granulocytes, and natural killer and antibody-de-

pendent cytotoxic cells (Ault and Springer, submitted). Presence on the latter cells was determined by FACS sorting of M1/70$^+$ and M1/70$^-$ fractions using F(ab')$_2$ M1/70 and testing for functional activity. Analogous experiments in the mouse are in progress.

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 (Pross and Eidinger, 1974). 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 (Springer et al., 1978). 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 (Springer, unpublished). Therefore, in order to increase the chance of obtaining macrophage-specific MAb, these 2 antigens were removed by an immunoadsorbent procedure as described below.

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 in the following way:

B6/J PEC ($8x10^8$ 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

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FIG. 1 (see next page).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 4d PEC. Cells were labeled with rat MAb, then FITC-rabbit anti-rat IgG as described previously (Springer et al., 1979). Mouse RBC, polymorphs, and macrophages appear at scatter intensities of 1, 4-5 and 7-10, respectively. GF SRBC = glutaraldehyde-fixed SRBC.

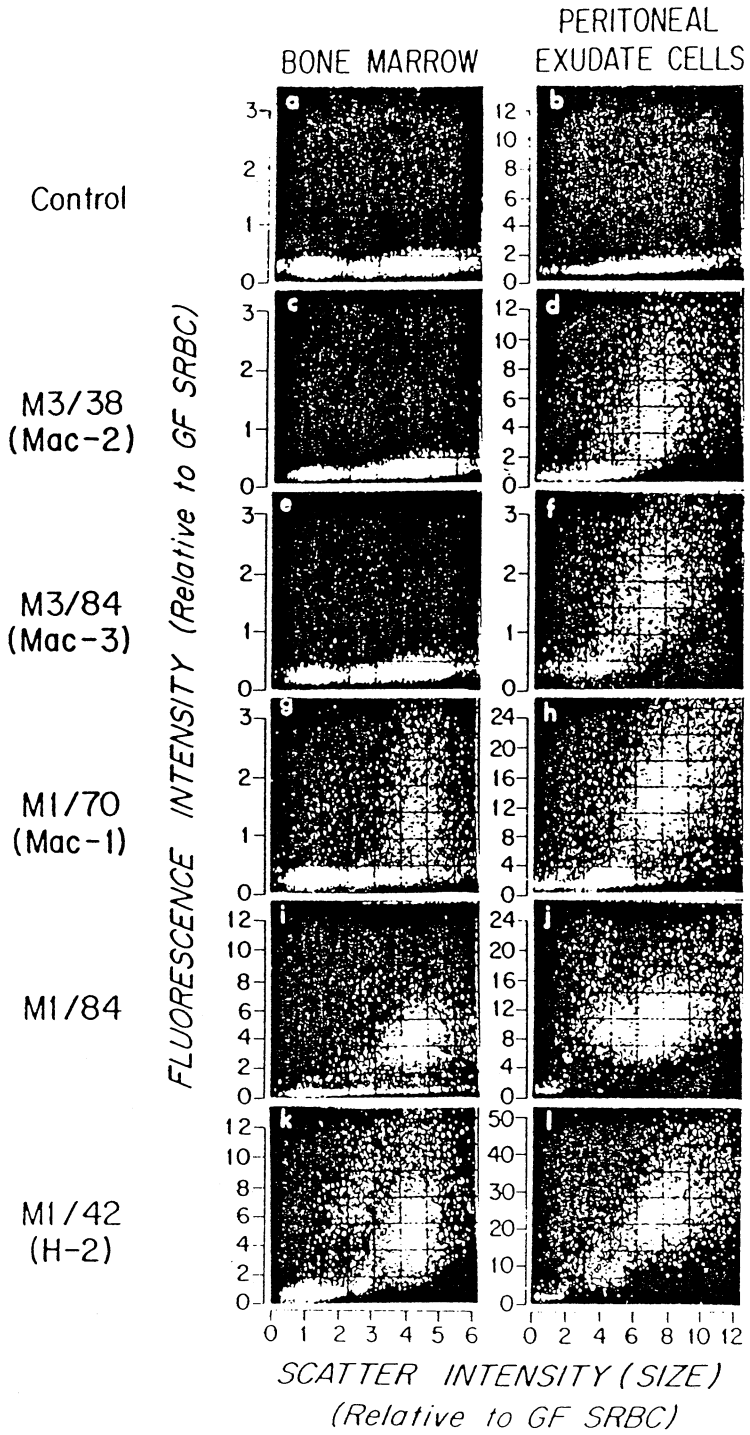

FIG. 1: For legend see previous page

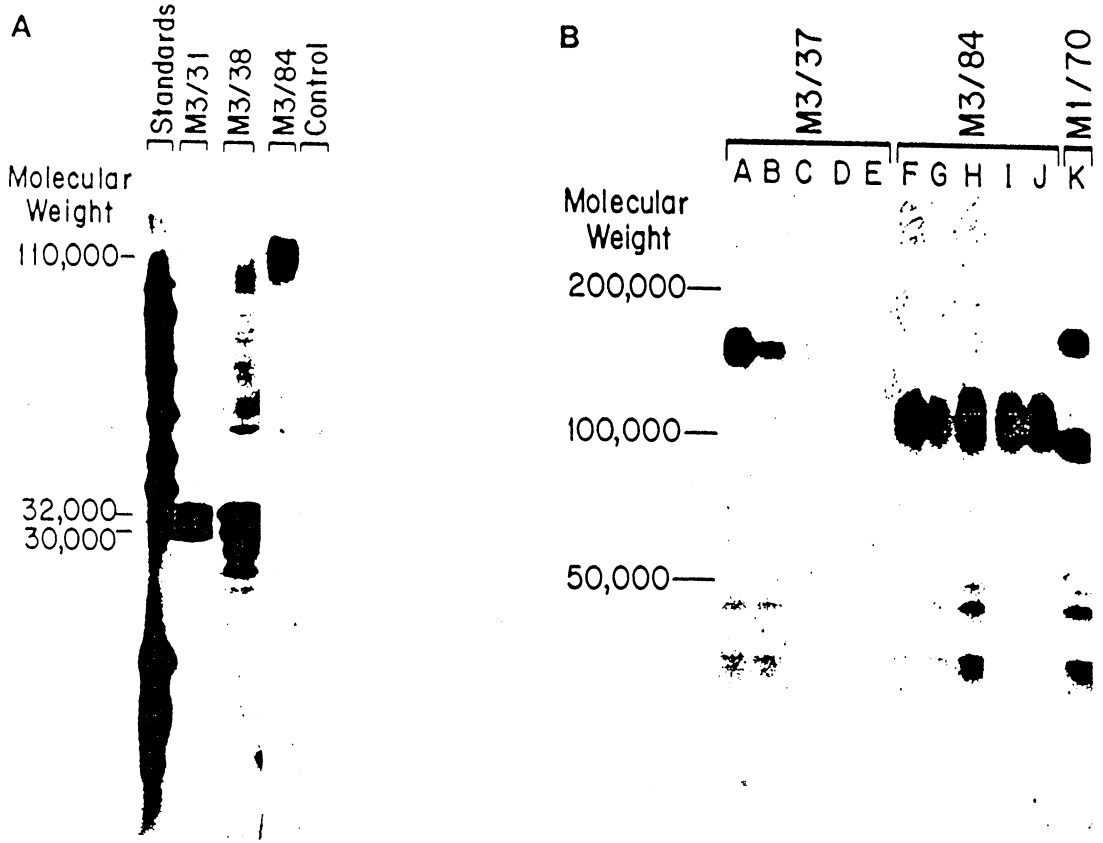

FIG. 2: For legend see overleaf

N_2 cavitated after 10 min at 600 psi. Membranes were prepared, solubilized with sodium deoxycholate (NaDC) and glycoproteins purified on L. culinaris lectin columns as previously described (Springer et al., 1977). 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 against 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 as described (Galfre et al., 1977), except that cells were aliquoted into 5x96 well 0.2 ml culture trays.

Hybrid culture supernatants were screened for binding to macrophages and all binders were then screened for immunoprecipitation of ^{125}I-labeled cell surface antigens. Five supernatants precipitated polypeptides of 190,000 MW and 105,000 MW which resemble those of Mac-1. Eleven cultures precipitated a 32,000 MW polpeptide, one a 110,000 MW, and another a 180,000 MW polypeptide. However, none of the cloned lines precipitated the 100,000 MW CLA polypeptide (Springer, 1980).

Three stable subloned lines were obtained by the cascade procedure (for brevity,sublone designations will be omitted). A 32,000 ME polypeptide (Mac-2), is precipitated by M3/31 and M3/38, while a 110,000 ME polypeptide (Mac-3) is precipitated by M3/84 (Fig. 2A). A 180,000 MW polypeptide (Mac-4) is precipitated by an uncloned line, M3/37 (Fig. 2B). (A cloned line, 54-1, has been independently obtained (Leblanc et al., 1980) which appears to recognize the same antigen as determined by comigration in SDS-PAGE. The cell distributions of all three antigens are very similar (Table 1). They are present on essentially all peritoneal exudate macrophages but on 0% of thymocytes and <10% of spleen cells. Mac-2, 3 and 4, in

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FIG. 2 (see previous page). 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 after 2-mercaptoethanol reduction (Springer et al., 1979).
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 sublone;
K: M1/70.15.1 subclone

contrast to Mac-1, are not expressed on bone marrow cells (0-1% positive)(Fig. 1). These results rule out expression on granulocytic precursors, but not on promonocytes, which constitute only 0.3% of bone marrow cells (van Furth, 1975).. Thus, Mac-2, 3 and 4 appear to be expressed on the monocytic line of differentiation at some stage after divergence from the granulocytic lines, while Mac-1 is found on both branches. Mac-1, 2 and 3 all appear to be expressed in lower amounts on macrophages than H-2 antigen (Fig. 1, note the changes in fluorescence scale).

The antigens described here all appear to be 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. 2A,data not shown for Mac-1). Many preparations of Mac-2 antigen show in addition to the 32,000 MW chain,4-fold less of a 30,000 MW chain. Much smaller amounts of slightly lower MW polypeptides precipitated by M3/38 but not M3/31 have also been seen (Fig. 2A). The working hypothesis is that the smaller polypeptides are degradation products bearing the M3/38 but not the M3/31 determinant. The M3/84 (Mac-3) 110,000 MW band is characteristically "fuzzy" after both ^{35}S-met and ^{125}I-labeling (Fig. 2B). Side-by-side comparison of the high MW Mac-4 (M3/37), Mac-3 (M3/84) and Mac-1 (M1/70) chains shows them all to have different MW's and thus, they are all clearly unique antigens (Fig. 2B). The stability of M3/84 and instability over time of M3/37 (Fig. 2B) illustrate the joys and sorrows, respectively, of hybridoma research. The MW and tissue distribution of these antigens differ from the macrophage Fc receptor II (Unkeless, this volume).Their relationship to the genetically defined Mph-1 antigen (Archer et al., 1974) is unknown.

Four different antigens have been defined in these studies which are present on macrophages but not lymphocytes, demonstrating the distinctiveness of macrophage cell surface architecture (Table 1). This also suggests that classical anti-macrophage sera, even after extensive absorption, recognize a complex of cell surface antigens.The Mac-1, 2, 3 and 4 antigens appear to be expressed on all thioglycollate-induced peritoneal macrophages (Fig. 1), and thus do not define qualitative subpopulations of these cells. However, macrophages exhibit greater heterogeneity in expression of Mac-1, 2 and 3 (Fig.1d,f,h) than in M1/84 or M1/42 (H-2) antigen (Fig. 1j,l) and thus the quantitative variations might define different subpopulations. Currently, the expression of these antigens on macrophages induced by other means and in different anatomical locations is being investigated. Absorption

studies with tissues such as liver are being carried out to determine whether other mononuclear phagocytes, e.g., Kupffer cells, also express these antigens. The MAb are also being used as probes to inhibit a panel of macrophage functions. In this way, it should be possible to link the molecular structures described here with specific macrophage cell surface activities.

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DISCUSSION

SILVERSTEIN: How does Springer's cross-reacting antibody to NK cells compare with the cross-reacting antibody for monocytes and NK cells described by Reinherz *et al*.?

SPRINGER: We do not have this information.

SILVERSTEIN: What is the evidence that mouse "lymphocytes" contain M1/70 antigen? Might not these cells be instead a subset of mononuclear phagocytes?

SPRINGER: They are termed "lymphocytes" based primarily on their size of the FACS and by their morphology displayed in

Wright stained preparations. The fact that they are M1/70 positive, however, suggests to me that they may be more closely related to myeloid cells than to lymphocytes.

KOREN: What is the evidence that the antibody described by Springer detects NK cells? What is the system he employs to detect NK?

SPRINGER: Cell sorting experiments have shown that the M1/70 positive cells were enriched for NK activity over stained unsorted cells while the M1/70 negative cells had no NK activity. The NK system used for those experiments was tumor K 562 and human blood lymphocytes.

UNKELESS: Would Springer tell us whether the anti-mouse antibody M1/70 can be used to immunoprecipitate an antigen from human cells? If that is the case, does the antigen resemble the 190K-105K pair of polypeptides isolated from mouse macrophages?

SPRINGER: We have been unable to precipitate any polypeptides from human cells. This is most likely due to the lower affinity of M1/70 for the cross-reacting human antigen than for the homologous mouse antigen. Monovalent ^{125}I-Fab' fragments do not bind to human monocytes whereas F(ab')$_2$ do. Precipitation probably involves a monovalent interaction. Thus, it appears that the antibody concentration (employed in both binding and precipitation experiments) is above the bivalent Kd, but significantly below the monovalent Kd for the human antigen.

HAYNES: I call to Springer's attention that there is a population of T-CLL patients who have a proliferation of T_G (FcR^+ for IgG) cells. Profile of these cells shows them to be low affinity E-rosette$^+$, Ia$^-$, 3AI$^+$ (35%), 100% OKT3$^+$, 100% OKT8$^+$, 15% OKT4$^+$ and 100% OKT6$^-$. They are negative for 4F2, our own monocyte marker. Thus, by these few criteria the T_G cells appeared to be more like "T" cells than "monocytes". Has Springer had occasion to study such patients?

SPRINGER: No, we have not; but I agree it would be of special interest to do so.

HOGG: Can Springer be certain that his M1/70 antibody is not reacting with human NK cells via the Fc receptor? Has he done any tests with pepsin digested antibody?

SPRINGER: We are quite certain on this point (Ault and Springer, manuscript submitted). M1/70 F(ab')$_2$ fragments have been prepared by us and contain no undegraded IgG as determined on overloaded SDS gels. The M1/70 F(ab')$_2$ labels

the same population both by FACS analysis and by sorting and testing for NK activity.

AUSTYN: What is Springers's source of primary macrophages? Would he tell us how many PMNs he finds in the thioglycollate-elicited peritoneal cells he works with? In our experience it has proved difficult to differentiate between PMNs, lymphocytes and sometimes even macrophages on the FACS. I would think that some autoradiographic analysis of binding of his monoclonal antibodies would be reassuring; has he done this?

SPRINGER: Our source of primary macrophages is 4 day thioglycollate-induced PECs. We find 1% PMNs in these populations.

We have had no trouble whatsoever, with our preparations, in distinguishing between macrophages and the smaller lymphocytes and PMN on the FACS. Autoradiography analysis of spleen cells with M1/70 has been done and published (Eur. J. Imm. 9, 301). Histiocytes, monocytes and granulocytes are labeled; with histiocytes showing more grains per cell, whereas lymphocytes and erythroid cells remain unlabeled.