ANTI-Mac-1 SELECTIVELY INHIBITS THE MOUSE AND HUMAN TYPE THREE COMPLEMENT RECEPTOR*

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Monoclonal antibodies (MAb)[1] have proven to be of great value in identifying the cellular lineages and subsets that give rise to the diversity of the immune system. Recently, interest has focused on the use of such antibodies to evaluate macrophage heterogeneity (1–5), and these reagents have added measurably to the information attained using heterospecific antisera (6). The first and perhaps best characterized of these antibodies, the rat anti-mouse M1/70 (anti-Mac-1) MAb, defines an antigen containing two polypeptides of 170,000 and 95,000 mol wt found on the surface of mouse macrophages, polymorphonuclear leukocytes (PMNL), and natural killer cells (1, 2, 7). The M1/70 MAb cross-reacts with human cells. Mac-1 has the same distribution in humans as in the mouse (8). To date, no function has been attributed to this antigen. The difficulty in ascribing function to antibody-defined cell surface structures has been a characteristic of studies on differentiation and cell type-specific antigens.

Current evidence indicates that cells of the myeloid lineage (mononuclear phagocytes and PMNL) bear two distinct cell surface receptors for fragments of activated C3 (9–12, and reviewed in 13). The type one complement receptor (CR_1) displays specificity for C3b and C4b (14–17). This receptor has been isolated and consists of a single polypeptide chain of 205,000 mol wt (14, 15). The type three complement receptor (CR_3) interacts with C3b inactivator cleaved C3b (C3bi) and further degradation products of C3bi termed α2D or C3d,g (16–22). To date, no information is available concerning the structure of CR_3. The type two complement receptor (CR_2), which interacts with C3d, is carried on lymphocytes but not on mononuclear phagocytes or PMNL (13, 23).

The purpose of this report is to present evidence that the anti-Mac-1 MAb selectively blocks the rosetting of C3bi-coated erythrocytes to murine macrophages and human PMNL. These results suggest that the Mac-1 antigen is intimately associated with or actually represents the type three complement receptor on these

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[1] *Abbreviations used in this paper:* C, complement; E, erythrocyte; E-IgG, erythrocyte-IgG antibody complex; E-IgM-C, erythrocyte-IgM antibody-complement complex; FCS, fetal calf serum; GVB, gelatin-containing veronal-buffered saline; HBSS, Hanks' balanced salt solution; MAb, monoclonal antibodies; PLL, poly-L-lysine, M_r >70,000; PMNL, polymorphonuclear leukocytes.

cells. This is one of the first cases in which a MAb-defined differentiation antigen has been associated with a specific cell surface function.

Materials and Methods

Murine Macrophages. Peritoneal exudate cells (PEC) were elicited from C3H/St mice (breeding colony of the Research Institute of Scripps Clinic, La Jolla, CA) or A/St mice (West Seneca Laboratories, Buffalo, NY) by injection of 1.5 ml of protease peptone 3 d before cell harvest. PEC were harvested with Hanks' balanced salt solution (HBSS) with 10 mM Hepes and 1% fetal calf serum (FCS), and macrophages were isolated by adherence (2 h, 37°C) of 2×10^5 PEC per ml RPMI 1640 medium with 5 mM Hepes and 5% FCS in 16-mm Diam wells of culture dishes (3524; Costar, Data Packaging, Cambridge, MA). Nonadherent cells were removed by three washes with HBSS, 10 mM Hepes.

Human PMNL. PMNL were isolated from human blood using the method of English and Anderson (24). Preparations exhibited >99% viability and contained >95% PMNL, as assessed by the Wright-Giemsa stain.

Purified human PMNL (2×10^5) in 1 ml HBSS were bound to 16-mm Diam flat-bottomed wells in multiwell tissue culture plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) that previously had been treated with 300 μl of poly-L-lysine (PLL) (70 μg/ml, M_r, >70,000; Vega Biochemicals, Tucson, AZ) as described (25). After the cells had adhered for 1 h at 37°C, the plates were washed and excess PLL blocked by addition of 0.5 ml HBSS containing 5 mg/ml ovalbumin. Before use, the adherent cells were washed with HBSS containing 1 mg/ml ovalbumin and 200 μg/ml soy bean trypsin inhibitor.

Preparation of Sheep Erythrocytes Bearing Antibody and Mouse Complement. Sheep erythrocytes (E) obtained from the Colorado Serum Company, Denver, CO, were washed three times in saline solution, resuspended to 2% (vol/vol) in HBSS with 5 mM Hepes and no FCS and then incubated with an equal volume of rabbit anti-sheep E antibodies for 30 min at 37°C. IgG antibodies and IgM antibodies (Cordis Laboratories, Miami, FL) were used at the highest dilution that had been shown to consistently give optimal rosetting (1:200 for IgG, 1:50 for IgM). After washing three times, the E-IgG and an aliquot of the E-IgM were set aside; the remainder of the E-IgM (2% in HBSS) was mixed with an equal volume of a 20% solution of freshly prepared A/St serum and incubated for 30 min at 37°C to produce complement (C)-coated E (E-IgM-C). These were also washed three times before use.

Preparation of Bovine E Bearing Human C3 Fragments. Bovine E were isolated from bovine blood preserved in Alsever's solution (Colorado Serum Company) and standardized to 10^9 cells/ml in gelatin-containing veronal-buffered saline (GVB). E bearing only C3b, C3bi, or C3d were prepared with purified human C components (26), as described elsewhere (17). Initial deposition of C3b onto the cells was accomplished by suspending 2×10^9 E in 200 μl C3 (3 mg/ml) and activating with 15 μg trypsin for 5 min at 23°C. Cells were washed two times with GVB, and a cell-bound C3 convertase formed by incubation of the cells for 3 min at 37°C with 200 μl of a mixture of factor B (120 μg/ml) and factor D (2.6 μg/ml) and 1.2 mM magnesium sulfate. Surface-directed C3 deposition was then started by addition of 10 μl 0.2 M EDTA, pH 7.2, and 50 μl C3 (3 mg/ml), and was allowed to proceed for 15 min at 37°C. Cells were washed and subjected to two more cycles of surface enzyme formation and C3b deposition. EC3b were finally washed in GVB and adjusted to 10^9 cells/ml. The number of C3b molecules bound per cell was determined either by using radiolabeled C3 during the cell preparation or by assessing the β1H globulin-binding capacity of the cells, which has been shown to be proportional to the number of cell-bound C3b. EC3b were converted to EC3bi by incubation with β1H (40 μg/ml) and C3b inactivator (8 μg/ml) for 30 min at 37°C. At the end of the reaction, cells were washed and readjusted to 10^9/ml. EC3d were produced from EC3bi by incubation of the latter with 10 μg/ml trypsin for 15 min at 37°C. The functional state of cell-associated C3 fragments was characterized as described (25). Briefly, cell-bound C3b was capable of producing positive immune adherence reactivity with human erythrocytes, of forming the alternative pathway C3/C5 convertase with factors B and D and magnesium ions, and of binding β1H. Cells bearing C3bi were lacking these three functions but exhibited the unique ability to be agglutinated by purified bovine conglutinin in the presence of calcium ions. Cells bearing C3d lacked all the above functions but retained 10% of the radioactivity previously associated with

C3b. A minimum of 100 C3b molecules or 500 C3bi molecules could be detected per cell by the functional tests.

Rosette Analysis. Adherent murine macrophages were washed with serum-free HBSS before use. Adherent human PMNL were washed with HBSS containing 1 mg/ml ovalbumin and 200 μg/ml soy bean trypsin inhibitor before use. The antibodies to be evaluated for inhibition of rosetting (see below) were then added (at 10 μg/ml unless otherwise indicated) in a final volume of 0.5 ml HBSS. After a 10-min preincubation at room temperature, 50 μl of the 2% opsonized E suspension was then added and the culture trays centrifuged (2 min at 200 *g*) to initiate binding of E. Trays were left undisturbed at room temperature for an additional 30 min, after which the nonadhering E were removed by agitating the trays during five changes of medium. Monolayers were fixed in 1% glutaraldehyde in saline, and the percent of macrophages with more than five E was determined by observation on an inverted microscope.

Blocking Antibodies. In most experiments, hybridoma culture supernatants diluted to the specified concentration were used as the source of MAb. Concentrations were determined by Mancini radial immunodiffusion against anti-Fab. The M1/70 rat anti-mouse Mac-1 MAb, γ2b kappa, 106 μg/ml (1), the purified M1/70 IgG, and the $F(ab')_2$ fragment were prepared as previously described (8). The 11-4.1 anti-H-$2K^k$ MAb (27), γ2a, 55 μg/ml, was used as a control in most experiments. The MAb used in Table I ranged from 23–235 μg/ml in the culture supernatant and have been previously described: M1/42 (28); M1/84, M1/89.18, and M1/9.3 (29); M5/111, M7/21, M7/83 (30); and M17/4.[2]

Results

Selective Inhibition by Anti-Mac-1 of Murine Macrophage C Receptor Function. Our goal in the present study was to evaluate the possible relationship of the Mac-1 antigen to a structure on the macrophage surface of known physiologic function. We initiated our investigation by evaluating the effect of anti-Mac-1 on the binding of E-IgG and E-IgM-C to the macrophage Fc and C3 receptors. After a 10-min preincubation with anti-Mac-1, anti-H-2K, or medium alone, appropriately opsonized E were adhered to the macrophage monolayers. The data in Fig. 1 indicate that, while neither anti-Mac-1 nor anti-H-2K had an appreciable effect on E-IgG binding, anti-Mac-1 completely blocked E-IgM-C binding, whereas anti-H-2K was without effect. Complete inhibition of E-IgM-C rosettes by anti-Mac-1 was seen at all concentrations of the sensitizing IgM antibody. In contrast, anti-Mac-1 had little effect on E-IgG rosettes, even at suboptimum IgG concentrations, showing that the selective effect on E-IgM-C rosettes was not related to the avidity of macrophages for opsonized E.

Anti-Mac-1-mediated inhibition of E-IgM-C binding to murine macrophages ranged from 71–100% in eight experiments conducted independently in our two separate laboratories. In these experiments, a very limited effect, ranging from 2–24% inhibition, was also seen in the E-IgG binding protocol. To distinguish a weak but potentially specific inhibition of E-IgG binding by anti-Mac-1 from a nonspecific competition by the Fc portion of anti-Mac-1 for the Fc receptor, we compared the inhibition of E-IgG and I-IgM-C binding after incubation with intact or $F(ab')_2$ anti-Mac-1. The data shown in Fig. 2 reveal that the weak inhibition of E-IgG mediated by intact anti-Mac-1 was totally absent in the $F(ab')_2$ preparation. However, the cleavage of the Fc portion of the antibody had no effect on its capacity to block E-IgM-C. Thus, anti-Mac-1 showed an absolute preference for blocking C receptor- vs. Fc receptor-mediated binding.

[2] Sanchez-Madrid, F., D. Davignon, E. Martz, and T. A. Springer. 1982. Functional screening for antigens associated with mouse T lymphocyte-mediated killing yields antibodies to Lyt-2,3 and LFA-1. Manuscript submitted for publication.

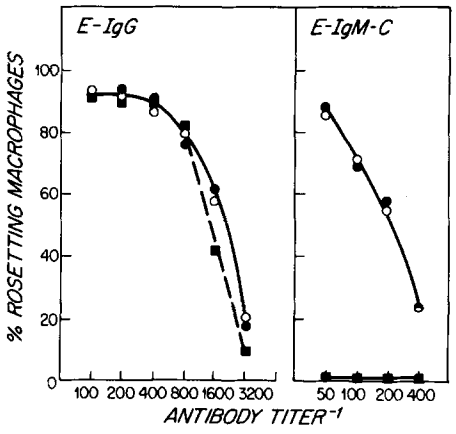

FIG. 1. Anti-Mac-1 blocks binding of E-IgM-C but not E-IgG. Peptone-elicited mouse peritoneal macrophages were exposed to anti-H-2K (●) or anti-Mac-1 (■), both at 10 μg/ml, or medium alone (○) for 10 min at room temperature. E opsonized with the indicated IgG or IgM antibody concentrations were then added. After centrifugation of E on to the macrophages, binding continued for 30 min at room temperature. Unbound E were removed by washing, and macrophages with more than five E bound were scored as positive.

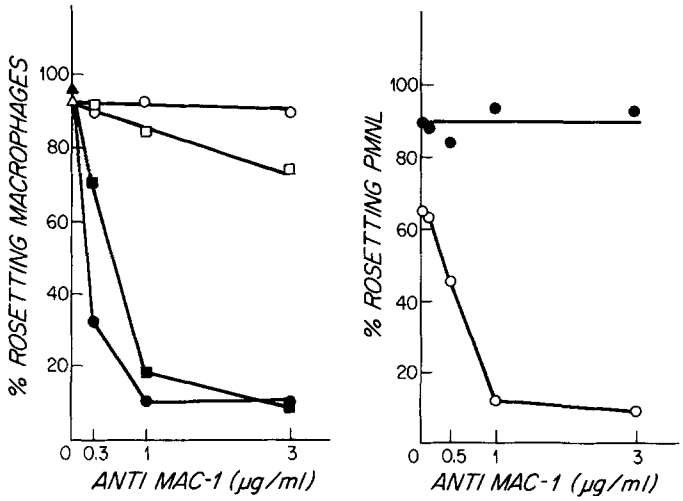

FIG. 2. (Left) Effect of intact or F(ab')$_2$ anti-Mac-1 on binding of opsonized E. Macrophages were preincubated with antibodies and rosetted with opsonized E, as in Fig. 1. Different concentrations of intact (□, ■) or F(ab')$_2$ (○, ●) anti-Mac-1 were compared with controls without antibodies (△, ▲) for blocking E-IgG (open symbols) or E-IgM-C (closed symbols).

FIG. 3. (Right) Inhibition of EC3bi but not EC3b rosettes by treatment of human PMNL with anti-Mac-1. Adherent human PMNL were incubated as described in Materials and Methods for 30 min at 37°C with designated doses of anti-Mac-1 or buffer in a total volume of 300 μl. After incubation, 100 μl of EC3b (●) or EC3bi (○) (5×10^7 cells/ml) were added and cell-cell contact initiated by centrifugation for 5 min at 100 *g*. Rosette formation was allowed to proceed for 30 min at 37°C. Cells were washed and rosettes were fixed by treatment with 1% glutaraldehyde and then scored by microscopic analysis.

We next evaluated a panel of MAb to determine whether other antibodies could also block rosetting (Table I). All antibodies except anti-LFA-1 were known to bind to macrophages, and anti-H-2 and the M1/84 anti-pan-leukocyte antibodies bound

TABLE I

*Only Anti-Mac-1 Blocks E-IgM-C Rosetting**

Blocking MAb	Percent rosetting macrophages	
	E-IgG	E-IgM-C
—	93	96
Anti-Mac-1 (M1/70, γ2b)	72	12
Anti-H-2 (M1/42, γ2a)	91	95
Anti-H-2 (M7/21, γ2b)	71	97
Anti-Ly5 (M1/89.18, γ2b)	82	94
Anti-Ly5 (M1/9.3, γ2a)	89	98
Anti-Lgp 100 (M7/83, γ2b)	92	96
Anti-pan-leukocyte (M1/84, γ2a)	93	94
Anti-pan-leukocyte (M5/111, γ2a)	86	97
Anti-LFA-1 (M17/4, γ2a)	95	87

* After adherence, peptone-elicited macrophages were incubated with the designated antibodies (5 μg in 0.5 ml) for 10 min at room temperature. Opsonized E (50 μl) were then added and centrifuged onto the macrophages. After a 30-min incubation at room temperature, unbound E were removed by several changes of medium and the macrophages fixed in 1% glutaraldehyde before quantitating binding. Positive cells were those with more than five E.

TABLE II

*Anti-Mac-1 Inhibits Murine Macrophage EC3bi Rosettes**

Opsonized erythrocytes	Control	Anti-Mac-1	
	Percent rosetting macrophages	Percent rosetting macrophages	Percent inhibition
E-IgM	3.8	5.1	0
E-IgM-C	97.6	22.9	77
EC3b	41.6	44.6	0
EC3bi	45.8	12.8	72

* Rosette analysis and rosette inhibition were performed as outlined in Table I, except E bearing either C3b or C3bi (86,000 C3b/E) were also used.

to a similar number of sites per cell as anti-Mac-1 (3). Of the nine antibodies tested, several showed slight inhibition of E-IgG binding. However, only anti-Mac-1 was able to inhibit E-IgM-C binding.

Selective Inhibition of CR_3-mediated Rosetting by Anti-Mac-1. The data presented thus far indicated that treatment of murine macrophages with anti-Mac-1 specifically abrogated the formation of rosettes with C-coated particles. E sensitized with serum as the source of C bear C3 mainly in the form of C3bi and C3d (13). Because macrophages bear receptors for C3bi but not C3d, this suggested that CR_3 (specific for C3bi) was blocked by anti-Mac-1. To more rigorously test which receptor was blocked, we prepared E bearing only homogenous fragments of C3 (C3b, C3bi, or C3d) by using highly purified proteins of the human alternative pathway. The number of C3 fragments bound per cell was ~75,000–100,000. Table II indicates that E bearing only human C3b or C3bi form rosettes with murine macrophages. The percentage of rosette-positive cells (41.6 and 45.8%, respectively) was significantly

reduced from the percentage that rosetted with E-IgM-C (97.6%). This difference is presumably a reflection of the lower affinity that exists between human C3 fragments and murine C3 receptors. In other experiments (data not shown), rosetting could not be shown between murine macrophages and EC3d. Pretreatment of the macrophage population with anti-Mac-1 caused a 77% reduction of EAC rosettes and a 72% decrease in rosettes formed with Ec3bi; no decrease was observed in C3b-mediated rosette reactions.

Because anti-Mac-1 is cross-reactive, its ability to inhibit human C receptors was also tested. Rosette inhibition analysis was performed with purified human PMNL and E bearing homogeneous human C3 fragments. In the controls, 90% of PMNL formed CR_1-dependent rosettes with EC3b, whereas 66% formed CR_3-mediated rosettes with EC3bi. Fig. 3 shows that anti-Mac-1 inhibited CR_3 rosetting in a dose-dependent fashion. Maximum inhibition approached 90%, and anti-Mac-1 at 0.8 μg/ml was sufficient to inhibit the rosette reaction by 50%. In contrast to this result, anti-Mac-1 was devoid of inhibitory activity toward CR_1-dependent rosettes. Even at 3 μg/ml of anti-Mac-1, the percentage of cells forming rosettes with EC3b remained unchanged from the buffer control.

Discussion

The results of this study indicate that anti-Mac-1 was unique among a panel of MAb known to bind to macrophages in being able to block E-IgM-C binding. Using particles that carried defined and homogeneous fragments of C3 on their surface, we showed further that anti-Mac-1 specifically blocked CR_3-mediated rosetting. No measurable inhibition of CR_1- or Fc receptor-dependent rosetting could be found, even at doses of anti-Mac-1 that were 5 to 10 times in excess of those that abrogated CR_3 rosetting. The most likely interpretation of these findings is that the Mac-1 antigen is the CR_3. However, the possibility exists that anti-Mac-1 recognizes a distinct structure that is intimately associated with CR_3 or that promotes CR_3 function.

C3 is the key component in the classical and alternative C pathways (reviewed in 26). When C3 is activated by cleavage to C3b, an internal thiolester bond is labilized (31–33). C3b can then become covalently attached to cell surfaces by acylation of surface component hydroxyl or amino groups by the thiolester. C3b is short lived under normal physiological conditions. It is rapidly cleaved to C3bi (inactive) by the concerted action of the serum components β1H and C3b inactivator and then further degraded to a form denoted α2D or C3d,g (19–22). α2D is converted to C3d at a much slower rate by serum proteases such as plasmin. Sensitization of E with IgM and C5-deficient serum under the conditions used here results in C3 essentially all in the C3bi form (13).

Three types of cell surface C receptors (CR) with specificity for C3 fragments have been described (12–17). CR_1 is specific for C3b and C4b. CR_2 binds to C3d and to the C3d portion of C3bi, but not to C3b (13, 23). CR_3 binds to C3bi (16–18). At present, it is not known whether CR_3 shows specificity for C3bi or for its degradative products, C3d,g or α2D (19–22). CR_2 is not expressed on macrophages or granulocytes, which are the cells under study in this report. CR_2 is restricted to B lymphocytes and is a glycoprotein of 72,000 M_r (23). CR_1 is a glycoprotein containing a single 205,000 M_r polypeptide (14). CR_1 activity on erythrocytes, lymphocytes, monocytes. macro-

phages, and neutrophils appears to be mediated by a common structure, as shown by blockade with antibody to the purified 205,000 M_r receptor.

CR_3 was only recently distinguished from CR_2 by the discovery that CR_3 binds C3bi but not C3d, whereas CR_2 binds both C3d and C3bi (13). CR_3 is present on monocytes, macrophages, and PMNL (13) and on 6–10% of human blood lymphocytes (18, 34). This is in good agreement with the distribution of Mac-1. Mac-1 is present on monocytes/macrophages, granulocytes, and on ~10% of human blood "lymphocytes." This lymphocyte subset shows greatly enriched natural killer and antibody-dependent cellular cytotoxic (ADCC) activity (8). Further, cells mediating ADCC have been reported to be enriched for the C3bi receptor (34). Kidney glomerular epitheloid cells are reported to express both CR_1 and CR_3 (12). However, we were unable to test whether anti-Mac-1 blocked the glomerular CR_3 because, in our hands, EC3b but not EC3bi bound to kidney glomeruli (unpublished observations). Kidney is negative for Mac-1 by absorption (2) and by immunoperoxidase straining of thin sections.[3]

We are in the process of purifying sufficient Mac-1 to evaluate whether C3bi and Mac-1 antigen interact directly. If successful, these experiments would confirm the identity of Mac-1 and CR_3. Mac-1 would then be one of the very few differentiation or cell-specific antigens identified by antibodies to which a specific function has been attributed. Moreover, such a finding would immediately shed light on the structure of CR_3 because we know that Mac-1 contains two noncovalently associated glycoprotein subunits, an α subunit of 170,000 M_r and a β subunit of 95,000 M_r (35). Because this is a large molecule, it is to be expected that not all MAb to it would block the complement-binding site.

The finding that anti-Mac-1 blocks CR_3-mediated rosetting is particularly intriguing because antibodies to a structurally related antigen on lymphocytes, LFA-1, block cytolytic T lymphocyte-mediated killing (30, 36). Anti-LFA-1 MAb block killing by binding to cytolytic T lymphocyte effectors and inhibiting their adhesion to target cells (37). LFA-1 contains noncovalently associated α and β subunits of 180,000 and 95,000 M_r (35). The M1/70 and M7/14 monoclonal antibodies defining Mac-1 and LFA-1 do not cross-react; and, as expected, anti-LFA-1 had no effect on C3bi-mediated rosetting (Table I). However, some antigenic determinants are shared between Mac-1 and LFA-1 (35, 38). Tryptic peptide mapping has shown the LFA-1 and Mac-1 β subunits are highly homologous, whereas the α subunits are distinct and appear to be products of different genes (35, 38). However, homology between the α subunits at the level of amino acid sequence has not been ruled out and appears likely by analogy to other protein families that have shared or homologous subunits. The structural homology between Mac-1 and LFA-1 suggests that there might be similarities among the molecular mechanisms underlying recognition of foreign cells by macrophages and T lymphocytes.

Summary

Anti-Mac-1 (M1/70), a rat monoclonal antibody that reacts with mouse and human macrophages, polymorphonuclear leukocytes (PMNL), and natural killer cells, selectively inhibited complement receptor-mediated rosetting by murine mac-

[3] Flotte, T., T. A. Springer, and G. J. Thorbecke. 1982. Dendritic cells and macrophage staining by monoclonal antibodies in tissue sections. Manuscript submitted for publication.

rophages and human PMNL. Preincubation of macrophages with anti-Mac-1 inhibited formation of rosettes with sheep erythrocytes bearing IgM antibody and murine C3 fragments. No inhibition was observed when other monoclonal antibodies that react with macrophages (such as anti-Ly5, anti-H-2, or anti-pan-leukocyte) were tested at 10-fold higher concentrations. Anti-Mac-1 did not affect macrophage Fc receptor-mediated rosetting. Erythrocytes bearing homogeneous human C3 fragments C3b (EC3b) or C3bi (EC3bi) were used to test the specificity of the murine macrophage and human PMNL complement receptor inhibited by anti-Mac-1. In both cases, anti-Mac-1 inhibited CR_3-mediated rosetting of EC3bi but not CR_1-dependent rosetting of EC3b. The results show that Mac-1 is either identical to CR_3 or closely associated with CR_3 function. This is one of the first cases in which a monoclonal antibody-defined differentiation antigen has been associated with a specific cell surface function.

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