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M5, a phosphoinositol-linked human myelomonocytic activation-associated antigen

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SUMMARY

When investigating the previously described monoclonal antibody (MoAb) VIM-5, raised against THP1 cells and binding to human monocytes and granulocytes, we found that the antigen detected by this antibody, designated M5, becomes very strongly expressed on monocytes after overnight culture with phorbol myristate acetate (PMA) or lipopolysaccharide (LPS) but not with recombinant human interferon-gamma (rhIFN-γ). Granulocytes stimulated with formyl-methionyl-leucyl-phenylalinine (FMLP) become negative for binding VIM-5. Immature granulocytes from bone marrow do not express M5, thus its expression on granulocytes is differentiation linked. The antigen bound by VIM-5 is sensitive to hydrolysis by phosphoinositol-specific phospholipase C (PI-PLC). The immunoprecipitated M5 antigen on monocytes is a broad band, with a peak of 50 kD (unreduced) and two bands of 53 kD and 44 kD (reduced). We have therefore detected an antigen that is up-regulated on stimulated monocytes but, conversely, down-regulated on FMLP-stimulated granulocytes.

Keywords phosphoinositol-linked myelomonocytic activation antigen

INTRODUCTION

The mononuclear phagocyte system, composed of monocytes and tissue macrophages, is a dynamic cellular system with various functional capabilities, including phagocytosis, antibody-dependent cellular cytotoxicity, secretion of macromolecules and antigen presentation. To exert their full functional potential, mononuclear phagocytes, like other cells, including lymphocytes, have to become activated, and some agents have already been described such as interferon-gamma (IFN-γ; Murray, Spitalny & Nathan, 1985), lipopolysaccharide (LPS; Liu *et al.*, 1985) and granulocyte–macrophage colony-stimulating factor (Weiser *et al.*, 1987), that can activate monocytes and macrophages to produce mediators such as tumour necrosis factor (de Titto, Catterall & Remington, 1986), interleukin-1 (Liu *et al.*, 1985) and reactive oxygen intermediates (Johnston, Godzik & Cohn, 1978).

From studies of human lymphocytes, we know that activation of T and B cells can be associated with profound changes in the surface antigen composition of these cells, for example new antigens and significant up-regulation of weakly expressed antigens have been described on the T lymphocyte CD2 structure (Holter *et al.*, 1986a) and also the T lymphocyte activation inducer molecule (Cebrian *et al.*, 1988) after activation with phorbol myristate acetate (PMA). For human monocytes, the up-regulation of weakly expressed or the appearance of new antigens detected by MoAb has been described for Fc receptors (Guyre, Marganelli & Miller, 1983), HLA class II (Basham & Merrigan, 1983), CD25 (Holter *et al.*, 1986) and Mo3e (Todd *et al.*, 1985). Here, we describe an antigen present on human monocytes that is up-regulated after stimulation with PMA or LPS whereas, on granulocytes, stimulation with FMLP results in loss of expression.

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MATERIALS AND METHODS

Preparation of normal blood cells

The mononuclear cell fraction of human peripheral blood and bone marrow specimens was separated by buoyant density gradient centrifugation (Boyum, 1968). The non-T lymphocyte fraction of peripheral blood leucocytes was purified by formation of rosettes with sheep erythrocytes. Approximately 25–35% of the mononuclear cell fraction did not form rosettes and were used as the monocyte-enriched fraction. Granulocytes were prepared from peripheral blood by buoyant density gradient centrifugation on Polyprep (Nycomed, Oslo, Norway). Bone marrow specimens were obtained with informed consent from the donor.

Stimulation of peripheral blood cells

Non-rosette forming mononuclear cells and granulocytes were cultured overnight in plastic flasks (Nunc Intermed, Denmark) at a concentration of 10^6 per ml in 20 ml of RPMI-1640 plus 10%

fetal calf serum (FCS) with or without other added stimulating agents, as indicated. Granulocytes were also stimulated with FMLP (Sigma, St Louis, MO) for 30 min at 37°C as described by Fischer, Majdic & Knapp (1987). After all stimulations, monocytes and granulocytes, after stimulation with FMLP, were greater than 90% viable by trypan blue exclusion.

Phenotypic analysis of cell lines

Reh 6 (pre-B cell), Daudi (B cell), Molt 4 (T cell), Hut 102 (HTLV-1 T cell derived cells), KG1 (myeloid), KG1a (myeloid), Hel (erythroid), HL60 (myeloid), U937 (monocytoid) and THP1 (monocytic) cell lines were cultured with or without 10^{-6}M PMA (Sigma) in RPMI-1640 plus 10% FCS for 24 h prior to harvesting and analysis.

Immunofluorescence assay

Cells were analysed by indirect immunofluorescence assay, essentially as described by Gadd & Ashman (1983). The cells were incubated in the presence of 10% heat-inactivated serum to blockade non-specific Fc receptor binding. Ethidium bromide in DPBS (1 μg/ml) was added to the cells to detect dead cells that take up this dye and fluoresce red. The cells were analysed for fluorescent staining with a flow cytometer (FACS 440; Becton-Dickinson, Mountain View, CA) and red fluorescent dead cells excluded. The monocyte and granulocyte populations were gated according to size and morphological criteria.

Phosphoinositol-specific phospholipase C (PI-PLC) hydrolysis of cells

PI-PLC derived from *B. thuringiensis* was incubated at a 1/500 dilution with fresh and cultured monocytes at 10^6/ml in DPBS for 30 min at 37°C using the method of Low *et al.* (1988).

Monoclonal antibodies

Monoclonal antibodies were used at a final concentration at 10 μg/ml unless otherwise indicated. MoAbs used included VIM-5 (IgG1 isotype), VIM 13 (IgM; CD14), VIFcR III (IgM; CD16), VID 1 (IgG1; HLA class II monomorphic), 32·2 (IgG1: FcRI), VIAP (IgG1; anti-alkaline phosphatase) and VIT200 (IgG2a: CD45). All MoAbs were raised in our laboratory, except for 32·2 which was a kind gift from Dr Guyre of Dartmouth Medical School, NH.

Analysis of M5 antigen molecular weight

Monocytes were surface labelled with $Na^{125}I$ (New England Nuclear, Boston, MA) essentially as described by Holter *et al.* (1986). Briefly, 100 μl of lactoperoxidase (170 U/ml; Sigma), 25 μl of 0·03% H_2O_2 and 2 mCi $Na^{125}I$ were added to 4×10^7 cells suspended in 300 μl DPBS. After 5 min at room temperature, the same amount of lactoperoxidase and H_2O_2 were added. After incubation a third time with H_2O_2, the reaction was stopped by washing three times with DPBS-1% BSA-10 mM KI. Radiolabelled cells were lysed in 0·8 ml lysis buffer (20 mM NaH_2PO_4, 2 mM EDTA, 2 mM EGTA, 2 mM NaF, 2 mM PMSF, 0·2% sodium deoxycholate, 1% NP40 and 0·5% CHAPS) pH 8·1. For PI-PLC digestion, 2×10^7 monocytes suspended in 200 μl DPBS were surface labelled with 2 mCi $Na^{125}I$, as described above. Radiolabelled cells were treated with PI-PLC according to the previously described protocol. After the incubation step, we carefully removed the cells and used the supernatants for immunoprecipitation. Cell lysates or enzyme-treated supernatants were then incubated with monoclonal antibody immunosorbents at 4°C for 4 h. Samples were analysed by SDS-PAGE and autoradiography.

RESULTS

Surface antigen phenotype of blood cells

The mAb VIM-5 was found to bind weakly to the monocyte and granulocyte fractions from whole blood (Fig. 1). Blood lymphocytes were negative. In the mononuclear cell fraction of bone marrow, monocytes were positive, whereas immature granulocytes of low density were almost completely unstained (Fig. 2).

Expression of M5 on stimulated cells

Monocytes cultured overnight in RPMI-1640 with 10% FCS bound VIM-5 more strongly than freshly isolated monocytes. This observed increase in expression of M5 antigen could not be inhibited by incubation with polymyxin B (data not shown).

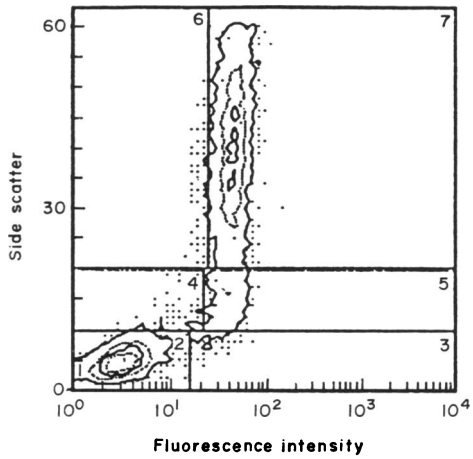

Fig. 1. Peripheral blood leucocytes were stained with FITC-labelled VIM-5 and analysed by flow cytometry. The lymphocyte, monocyte and granulocyte populations were gated according to size and granularity and lack of staining with non-antigen-specific MoAb VIAP.

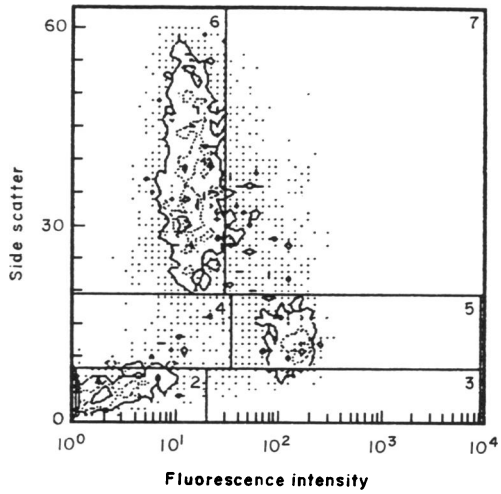

Fig. 2. The mononuclear cell fraction of normal bone marrow was stained by immunofluorescence assay with flow cytometric analysis using MoAb VIM-5. The lymphoid, monocyte and immature granulocyte fractions were gated according to size and granularity and lack of staining with non-antigen-specific MoAb VIAP.

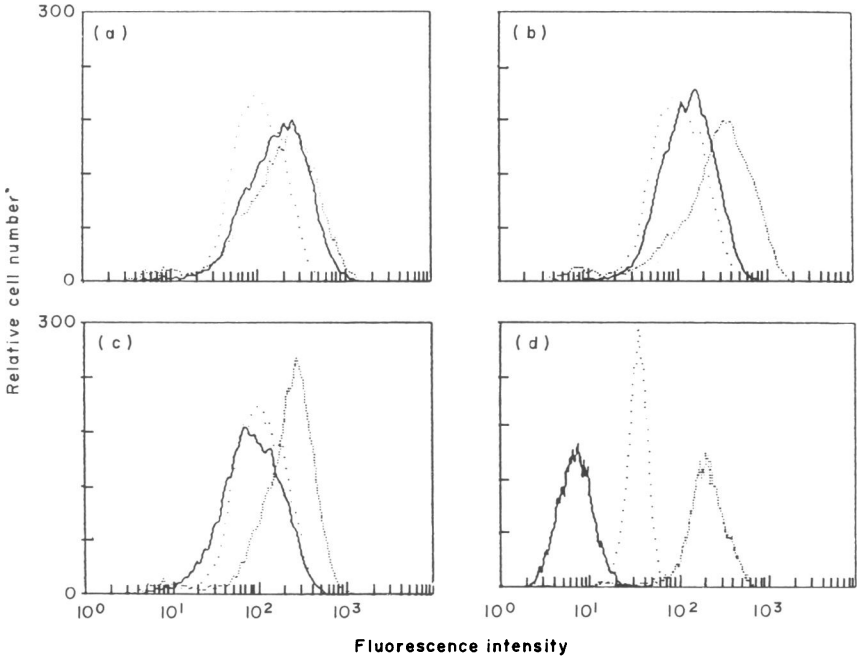

Fig. 3. The immunofluorescence profiles (a), (b) and (c) denote gated monocytes stained with VIM-5 after incubation for 22 h in the absence of a stimulus (· · · ·) or with a stimulating agent (——), which was, respectively, 10^{-9}M PMA, 10 μg/ml LPS and 1000 U/ml rhIFN-γ. Profile (d) denotes binding of VIM-5 to unstimulated granulocytes (· · · ·) and after stimulation with 10^{-7}M FMLP for 30 min at 37°C (——). The monocyte and granulocyte fractions after stimulation were greater than 90% positive with monocyte-specific MoAb VIM 13 (CD14) and granulocyte-binding MoAb VIFcR III (CD16), respectively (· · · ·).

Culture *in vitro* of monocytes for 22 h in the presence of PMA (10^9 N – 5×10^{-9}M) resulted in strong binding of VIM-5 (Fig. 3a). Stimulation of monocytes for 22 h in the presence of LPS (10 ng – 1/μg/ml) also resulted in stronger binding of VIM-5 (Fig. 3b). This LPS-induced stimulation of M5 antigen expression could be partially, but not completely, inhibited by co-incubation with polymyxin B (data not shown). Incubation of monocytes for 22 h with rhIFN-γ 1–1000 U/ml), which up-regulated binding of mAb VID 1 and 32·2 (data not shown), did not significantly alter expression of M5 antigen (Fig. 3c).

Normal granulocytes were weakly positive for M5 antigen, but after culture for 22 h without added stimulus showed weaker M5 expression (data not shown). Stimulation of granulocytes with rhIFN-γ (1000 U/ml), LPS (100 ng/ml) or PMA (10^{-9}M) for 22 h *in vitro* did not result in a significant change in M5 expression (data not shown). After stimulation with FMLP for 30 min, granulocytes became negative (Fig. 3d), showing that M5 antigen is rapidly down-regulated on granulocytes activated with FMLP *in vitro*.

As shown in Table 1, all lymphoid cell lines, including Molt 4, Reh 6, Hut 102 and Daudi, were negative for binding VIM-5. Myeloid cell lines bound VIM-5 variably, as some myeloid cell lines (HL60, HEL and U937) are negative but after stimulation with PMA are positive. THP1, which was constitutively positive for M5 antigen, became more strongly positive after stimulation with PMA. The myeloid cell lines KG1 and KG1a did not bind VIM-5 antibody and remained unchanged after stimulation with PMA.

Table 1. Analysis of VIM-5 binding to cell lines

	Immunofluorescence assay	
Cell line	minus PMA	plus PMA
Reh 6	–	–
Daudi	–	–
Molt 4	–	–
Hut 102	–	–
KG1	–	–
KG1a	–	–
HEL	–	±
HL60	–	+
U937	–	+
THP1	+	++

Cell lines were cultured with or without 10^{-6}M PMA for 24 h prior to immunofluorescence analysis.
++, very strong staining; +, strong staining; ±, weak staining; –, negative staining.

Monocyte surface antigen sensitivity to PI-PLC

Incubation of monocytes with PI-PLC resulted in significantly decreased binding of the MoAb VIM-5 and VIM 13 (Fig. 4a, b), though binding of these MoAb was not totally lost. Binding of mAb VIT200 was unaffected by PI-PLC incubation (data not shown), thus stripping of all antigens by contaminating proteases was not occurring.

Analysis of M5 molecular weight

Immunoprecipitation experiments with MoAb VIM-5 and lysates of ^{125}I-labelled freshly isolated human monocytes revealed a very broad protein band under non-reducing conditions of 40–60 kD (Fig. 5, lane 2). Under reducing conditions,

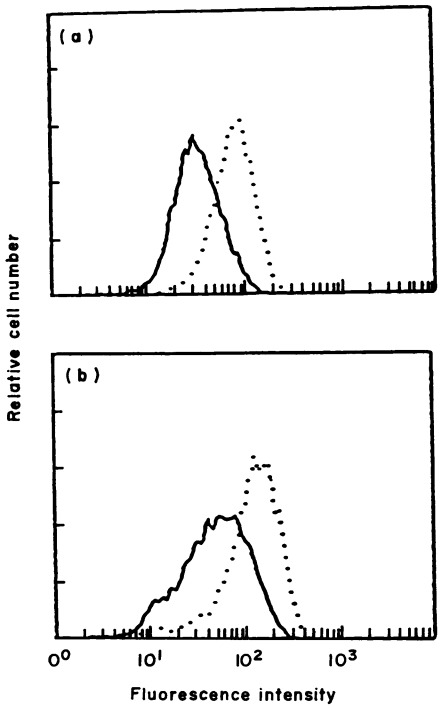

Fig. 4. Monocytes cultured for 22 h *in vitro* with (——) or without (· · · ·) a 1/500 dilution of PI-PLC for 30 min at 37°C and then analysed by immunofluorescence assay with flow cytometric analysis for binding MoAb VIM-5 (a) and VIM 13 (b).

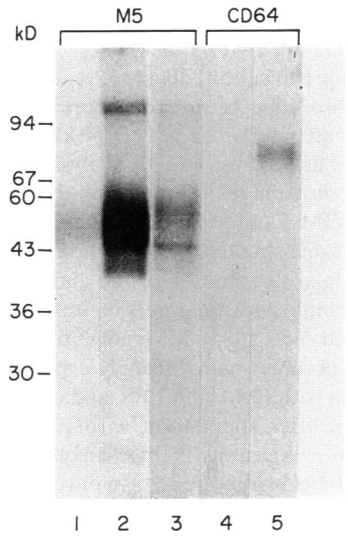

Fig. 5. Immunoprecipitates were analysed on a 12% SDS–PAGE gel. Lane 1 shows the precipitate of M5 from cell-free supernatants of PI-PLC-treated ^{125}I-labelled monocytes under non-reducing conditions. Lane 2 and 3 show the precipitate of M5 from cell lysates of ^{125}I-labelled monocytes under non-reducing and reducing conditions, respectively. As a control, an antibody binding to CD64 (FcRI) was used. Lane 4 contains immunoprecipitate from the cell-free supernatant of PI-PLC-treated monocytes, and in lane 5 the immunoprecipitate from cell lysates was run under non-reducing conditions. Proteins used as molecular weight markers were: phosphorylase B (94 kD), albumin (67 kD), catalase (60 kD), ovalbumin (43 kD), lactate dehydrogenase (36 kD) and carbonic anhydrase (30 kD), from Pharmacia, Uppsala, Sweden.

the band was resolved into two bands of 53 kD and 44 kD, respectively (lane 3). This finding suggests that the molecules detected have intramolecular disulphide bonds. In some experiments, a very faint band of 32 kD, which shifted under reducing conditions to 36 kD, could be observed (data not shown). PI-PLC treatment of ^{125}I-labelled monocytes and the immunoprecipitation with MoAb VIM-5 from the cell-free supernatant revealed, under non-reducing conditions, a 50 kD band (lane 1).

DISCUSSION

In 1984, a MoAb designated VIM-5 was raised against THP1 cells and was described to bind weakly to a protein antigen on blood monocytes (Knapp *et al.*, 1984). We further characterized this antigen, designated M5, for cellular distribution, functional association and biochemical characteristics. Analysis of M5 expression on blood cells showed that only monocytes and granulocytes expressed M5 (Fig. 1). Of the mononuclear cell fraction of bone marrow cells, only monocytes were significantly positive. Thus, M5 is a myelomonocytic-associated cell surface antigen, the expression of which is also linked to terminal differentiation on granulocytes.

We were interested to determine whether M5 antigen is expressed on myeloid cell lines. We found that the poorly differentiated cell lines KG1 and KG1a were negative, whereas THP1 cells with monocytic cell characteristics were constitutively positive.

Many activation-inducing agents have been described that alter cell surface expression of monocyte and granulocyte antigens *in vitro*, including hIFN-γ, LPS, PMA and FMLP (Guyre, Marganelli & Miller, 1983; Basham & Merigan, 1983; Liu *et al.*, 1985; Fischer, Majdic & Knapp, 1987). We stimulated monocytes and granulocytes with some of these agents to examine their effect upon M5 expression.

Stimulation of monocytes and granulocytes with rhIFN-γ LPS and PMA had different effects on monocytes and granulocytes. In monocytes but not granulocytes, PMA, and to a lesser extent LPS, but not rhIFN-γ caused up-regulation of M5 expression, whereas in granulocytes none of these stimuli had a significant effect. Culture of monocytes without added stimulus resulted in weak up-regulation of M5 expression. Granulocytes initially expressing low levels of M5 antigen after stimulation with FMLP lost all detectable M5 antigen. Thus M5 expression on monocytes is up-regulated by PMA and LPS but not rhIFN-γ, whereas in granulocytes these stimuli had no effect, but M5 expression is down-regulated by stimulation with FMLP. Some initially negative myeloid cell lines after stimulation with the potent cell activation-inducing agent PMA showed up-regulation of M5 expression (Table 1).

Immunoprecipitation of M5 (unreduced) showed a broad protein band of about 40–60 kD (peak 50 kD) and two bands of 53 kD and 44 kD (reduced) from monocytes in all experiments. The two immunoprecipitated protein bands shown under reducing conditions may represent different glycosylated forms. Expression of M5 antigen on monocytes was partially sensitive to hydrolysis with PI-PLC indicating that phosphoinositol-anchored and membrane integral forms may exist. The up-regulation of M5 expression by monocytes and myeloid cell lines

resembled that previously described for the Mo3e antigen (Todd *et al.*, 1985; Liu *et al.*, 1989). Furthermore, Mo3e (R. F. Todd III, personal communication), like M5, is also phosphoinositol linked. MoAb Mo3e has been reported previously to immunoprecipitate a broad band with a peak of 50 kD and a 75 kD band from monocytes using Western blot analysis (Liu *et al.*, 1989). The 50 kD protein band may be common to both antigens and therefore both VIM-5 and Mo3e antibodies may detect different epitopes on the same structure. Clearly though, differences exist between the cellular distribution of M5 and Mo3e, as Mo3e is not found on granulocytes and rarely on freshly prepared blood monocytes (Todd *et al.*, 1985). M5 is constitutively expressed on monocytes but becomes more strongly exposed on these cells after stimulation with PMA and LPS, and on granulocytes M5 becomes hidden after stimulation with FMLP, perhaps via activation of an endogenous PI-PLC enzyme. The functional significance of the M5 structure on myeloid cells is unknown but it may be a receptor or receptor ligand, a cytoadhesin, or a signal transducing structure, all of which have been previously reported for PI-linked structures, including CD16 (Huizinga *et al.*, 1988), LFA-3 (Seed, 1987) and CD14 (Macintyre *et al.*, 1989). This structure is of great interest as, along with FcRI and Mo3e, it is one of the few myeloid cell-specific activation-associated structures that have been reported to date.

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