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Characterization of a novel myeloid antigen regulated during differentiation of monocytic cells*

The monoclonal antibody HCl/6 generated against phorbol 12-myristate 13-acetate-treated U-937 cells recognizes a new cell surface antigen with a broad relative molecular mass ranging from 100 to 150 kDa. This antigen is also present on monocytes, platelets and endothelial cells and is weakly expressed by granulocytes. In contrast, it is absent from T, B and erythroblastoid cells. The antigen HCl/6 is also expressed by normal tissue macrophages in tonsil, lung and kidney, as well as in skin biopsies from pathologies such as sarcoidosis and lepromatous leprosy. The expression of the HCl/6 antigen is increased up to 5-fold when U-937 (promonocytic) and HL-60 (myelomonocytic) cell lines are stimulated with phorbol 12-myristate 13-acetate. Conversely, the expression of the HCl/6 antigen is down-regulated in monocytes upon treatment with interferon-γ. These findings are discussed in relation with other myeloid cell surface markers.

1 Introduction

The differentiation of monocytes is a multistep process characterized by major changes in their morphological and functional properties [1, 2]. These changes can correlate with the regulated expression of different cell surface antigens which are useful for the analysis of discrete stages of differentiation. A variety of agents including interferon (IFN)-γ, retinoic acid (RA), phorbol 12-myristate 13-acetate (PMA) and 1,25-dihydroxyvitamin D3 [1,25-(OH)2D3] are able to generate functionally different subpopulations and to regulate the expression of cell surface proteins of the monocytic lineage [3-7]. The existence of these subpopulations can be analyzed by using monoclonal antibodies (mAb) specific for surface antigens [8]. Thus, CR3 (CDlib or Mo1) is mainly present in circulating monocytes and granulocytes, whereas CD11c (p150,95) is highly expressed in tissue macrophages (MΦ) [9-11]. Also, the expression of CR1 (CD35), CR3 (receptor for C3bi), CD11c and FcRI (CD16) is rapidly increased when cells are treated with certain stimuli such as IFN-γ, PMA or the N-formyl-Met-Leu-Phe peptide [9-15].

The promonocytic cell line U-937 and the myelomonocytic cell line HL-60 have often been used to analyze the myeloid differentiation process [16-18]. Here, we have used the MΦ-like U-937 cells after differentiation in the presence of PMA as a model system to generate mAb against novel cell surface antigens of the myeloid family which are up-regulated after differentiation. Thus, we have obtained the mAb HCl/6 which recognizes a novel monocytic antigen of 100-150 kDa. The characterization of this antigen is described.

2 Materials and methods

2.1 Cell lines

U-937 (promonocytic), HL-60 (myelomonocytic), K-562 (erythroblastoid), Jurkat (T lymphocytic), JM (T leukemic) and JY (B lymphoblastoid) cell lines were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, penicillin, 100 U/ml and streptomycin (100 ng/ml) (Flow Laboratories, Rockwell, MD), in a 5% CO2 atmosphere at 37 °C. When necessary, cells were treated with PMA (10 ng/ml), 1,25-(OH)2D3 (10-8 M), IFN-γ (100 U/ml) or RA (100 ng/ml).

2.2 Preparation of leukocytes

Peripheral blood leukocytes were obtained from heparinized whole venous blood. After elimination of most erythrocytes by gravity sedimentation in the presence of 0.4% dextran, monocytes (Mo) and lymphocytes were separated from granulocytes by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Granulocytes were obtained from the pellet and separated from contaminant erythrocytes by a 20-s hypotonic lysis in distilled water. Mo were isolated by incubating the mononuclear cell fraction in a slightly hypertonnic medium followed by a second modified Ficoll-Hypaque density centrifugation [19]. Alternatively, Mo were purified by centrifugal elutriation as described by Figdor et al. [20].

Mo (3 × 106/ml) were incubated at 37 °C, 5% CO2 and 100% humidity in Teflon bags (Jansen's MNL, St. Niklaas, Belgium) in a modified Iscove's medium containing human albumin and supplemented with 2% autologous heat-inactivated serum [21]. After 2 days in culture, Mo were collected from the Teflon bags, washed, resuspended in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin, 0.02% sodium azide and kept on ice until the detection of surface membrane determinants. Platelets were obtained by density centrifugation [20].

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Abbreviations: mAb: Monoclonal antibody(ies) 1,25-(OH)2D3 or Vitamin D3; 1,25-Dihydroxycholecalciferol PMA: Phorbol 12-myristate 13-acetate RA: Retinoic acid IFN: Interferon Mo: Monocyte(s) MΦ: Macrophage(s) SF: Specific fluorescence
Recombinant IFN-γ (a gift from Dr. Adolf, Boehringer-Ingelheim, Vienna, Austria) was solubilized in RPMI 1640 containing 10% FCS and stored at -20°C, 1,25-(OH)2D3 (a gift of Dr. Uskokovic, Hoffmann-La Roche, Nutley, NJ) and RA (Sigma, St. Louis, MO) were stored at -20°C in ethanol. PMA (Sigma) was dissolved in dimethylsulfoxide to a concentration of 1 mg/ml.

### 2.3 Reagents

2.3.1 Ethanol

2.3.2 PMA

2.3.3 Ethylendiaminetetraacetic acid (EDTA)

2.3.4 Sodium chloride

2.3.5 Calcium chloride

2.3.6 Magnesium chloride

2.3.7 Lipopolysaccharide (LPS)

2.3.8 Fetal bovine serum (FBS)

2.3.9 Phosphate-buffered saline (PBS)

2.3.10 Phenylmethylsulfonyl fluoride (PMSF)

2.3.11 Sodium dodecyl sulfate (SDS)

2.3.12 Polyethylene glycol (PEG)

2.3.13 Cell culture media

2.3.14 Antibodies

2.3.15 Enzymes

2.3.16 Chromogenic substrates

2.3.17 Nuclear stains

2.3.18イメージングシステム

### 2.4 mAb

The mAb HCl/1 (anti-CD11c) [22], FG1/6 (specific for the transferrin receptor) [23], HF2/6 (anti-CD4) [24] and 8F3 (anti-CD13) have been generated in our laboratory. The mAb Bear 1 (anti-CD11b) and Bear 2 (anti-CD14) [25, 26] were kindly provided by Dr. Jan De Vries (UNICET, Lyon, France). The mAb Q5/13, which detects a determinant common to HLA-DR, -DQ and -DP molecules [27], was a gift of Dr. S. Ferrone (New York Medical College, New York, NY).

### 2.5 Production of the hybridoma HCl/6

BALB/c mice were injected i.p. with 15 × 10⁶ PMA-treated U-937 cells on days 48, 33 and i.v. on day 3 prior to fusion. Spleen cells were fused on day 0 with P3-X63Ag8.653 mouse myeloma cells at a 4:1 ratio according to standard techniques, distributed into 96-well plates (Costar, Cambridge, MA) and grown as described [22]. After 2 weeks, hybridoma culture supernatants were harvested and screened for binding to PMA-differentiated U-937 cells using the 125I-labeled rat anti-mouse x chain mAb 187.1 as second antibody [28]. The mAb HCl/6 was selected due to its enhanced reactivity for PMA-treated over untreated U-937 cells. This antibody is of the IgG1 subclass.

### 2.6 Radiolabeling, immunoprecipitation and electrophoresis

Cells were iodinated using chloroglycoluril (IODO-GEN, Sigma, [29]) and lysed in PBS, pH 7.4, containing 1% Triton X-100, 1% hemoglobin and 1 mM phenylmethylsulfonyl fluoride. Immunoprecipitation was carried out with protein A-Sepharose (Pharmacia; [22]) and samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis [30] and autoradiography with enhancing screens [31].

### 2.7 Flow cytometry

Flow cytometry analysis was performed using either an EPICS-C (Coulter Científica, Mostoles, Spain) or a FACScan (Becton Dickinson, Mountain View, CA) flow cytometer using logarithmic amplifiers [22]. Specific fluorescence (SP) was obtained by subtracting the peak channel number of the negative control from the peak channel number of the corresponding experimental sample.

### 2.8 Immunohistochemical staining of human tissue sections

Specimens of human tonsil, thymus, lymph node, brain, lung, skin and kidney were obtained by surgical procedures. Tissue sections were stained by an indirect one-step immunoperoxidase method [22, 32]. For the double immunoperoxidase-alkaline phosphatase staining, we followed the sequential method reported by Masson et al. [33]. After the development of the peroxidase reaction, sections were incubated with the second mAb, then with a rabbit anti-mouse IgG, followed by a third incubation with the alkaline phosphatase-anti-alkaline phosphatase mAb complex (Dakopatts, Glostrup, Denmark). Each incubation was followed by three washes with Tris saline. Finally, the alkaline phosphatase reaction was developed, yielding a dark blue precipitate which contrasts with the brown colour of the peroxidase reaction. Sections were mounted in buffered gelatin for microscopic examination.

### 3 Results

#### 3.1 Effect of several differentiation agents on the expression of the antigen recognized by the mAb HCl/6

The mAb HCl/6 was obtained by immunization with PMA-treated U-937 cells. The antigen recognized by HCl/6 is moderately expressed on untreated U-937 cells, but its expression is strongly increased after PMA-induced differentiation (Fig. 1). IFN-γ, 1,25-(OH)2D3 and RA were also used to induce differentiation of U-937 cells and the expression of HCl/6 and several other cell surface markers was analyzed by flow cytometry (Fig. 1). The highest expression of the antigen recognized by the mAb HCl/6 was obtained in the presence of PMA. A certain augmentation of the HCl/6 antigen was also detected with IFN-γ and 1,25-(OH)2D3 in contrast to the decrease observed in the presence of RA. Concomitantly with the loss of proliferation (data not shown), the expression of the transferrin receptor was markedly diminished in every differentiation protocol. The regulation of the HCl/6 antigen expression is accompanied by different antigenic changes depending on the differentiation agent used. In addition to HCl/6, PMA enhances the expression of CD11c and CR3 (CD11b) antigens and diminishes the expression of the CD4 antigen. In the presence of 1,25-(OH)2D3 and RA, CR3 expression is also increased but the CD11c antigen is almost unaffected. A feature common to RA and vitamin D3 but not to PMA is the marked decrease of the CD14 staining. On the other hand, CD4 expression was found to be affected only by RA and PMA. These results demonstrate that the expression of the antigen recognized by the mAb HCl/6 can be up-regulated by PMA, IFN-γ and 1,25-(OH)2D3. This regulation does not strictly follow any of the patterns shown by the antigenic markers used as controls. Kinetic studies in the presence of PMA demonstrated that the antigen HCl/6 shows the highest expression after 3 days in culture, similarly to the CD11c antigen (data not shown).

In vitro, IFN-γ induces activation of human Mo, which is associated with an increased expression of the high-affinity FcR for IgG (FcγRI) and major histocompatibility complex (MHC) class II antigens [15, 34, 35], as well as a decreased expression of CD14, CR3 and 63D3 antigens [36]. We then assayed the effect of IFN-γ on the expression of the HCl/6 antigen by human Mo (Table 1). Mo cultured in the presence of IFN-γ showed a marked decrease in the expression of HCl/6 as compared with the control culture. As expected, expression of MHC class II antigens increased during the culture of Mo but IFN-γ and IFN-α up-regulated further their expression.
Regulated expression of a novel myeloid antigen

Figure 1. Effect of different stimuli on the expression of the antigen recognized by the mAb HCl/6. Unstimulated and PMA- (3 days), vitamin D3-(5 days), IFN-γ-(5 days) or RA-(5 days) treated U-937 cells were incubated with Bear 1 (anti-CD11b), HCl/1 (anti-CD11c), Bear 2 (anti-CD14), HCl/6 (anti-CD4), FG1/6 (anti-transferrin receptor), 8F3 (anti-CD13) or HCl/6 mAb and assayed for indirect immunofluorescence in an EPICS-C flow cytometer as described in Sect. 2.7. Arrows indicate the increase or decrease in fluorescence after treatment of cells with the differentiation agents as compared to unstimulated cells.

Figure 2. Reactivity of the mAb HCl/6 with different cell types. Figures at the upper right corners represent the percentage of positive cells. (A) Untreated or PMA-treated U-937 (promonocytic), HL-60 (myelomonocytic), K-562 (erythroblastoid), Jurkat (T leukemic), JM (T leukemic) and JY (B lymphoblastoid) cell lines. (B) Peripheral blood granulocytes, lymphocytes, Mo and platelets. Cells were incubated with the HCl/6 mAb and assayed for indirect immunofluorescence in an EPICS-C flow cytometer as described in Sect. 2.7.

Table 1. Effect of IFN-γ and IFN-α on the expression of the antigen HCl/6 in Mo

<table>
<thead>
<tr>
<th>Addition</th>
<th>HCl/6 (%)</th>
<th>HLA-DR (%)</th>
<th>HCl/6 SF</th>
<th>HLA-DR SF</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>96</td>
<td>98</td>
<td>4.7</td>
<td>26</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>26</td>
<td>97</td>
<td>3.2</td>
<td>41</td>
</tr>
<tr>
<td>IFN-α</td>
<td>95</td>
<td>97</td>
<td>4.4</td>
<td>52</td>
</tr>
<tr>
<td>Control</td>
<td>96</td>
<td>93</td>
<td>5.1</td>
<td>9</td>
</tr>
</tbody>
</table>

a) Mo purified by the countercurrent elutriation method were incubated in the absence or in the presence of IFN-γ (100 U/ml) or IFN-α (100 U/ml) for 2 days at 37°C in 5% CO₂. Cells were stained with either HCl/6 or the anti-HLA-DR mAb Q5/13 and their fluorescence was analyzed in a FACScan flow cytometer.
b) SF values were obtained as described in Sect. 2.7.
c) A control sample kept at 4°C was also included for comparison.
No changes in the level of expression of the antigen HC1/6 were obtained in the presence of IFN-γ.

Other cell types were also assayed for reactivity with the mAb HC1/6 (Fig. 2). No staining was found with the erythroblastoid cell line K-562 nor with the lymphoblastoid B cell line JY and the T cell line JM. Similarly, a very weak reactivity was found with the T cell leukemic line Jurkat. In addition, granulocytes were weakly labeled by the mAb HC1/6, whereas peripheral blood Mo and platelets were clearly positive as opposed to the negative staining of lymphocytes. The relatively restricted expression of this antigen to cells of the myeloid lineage (U-937, HL-60, Mo and platelets) becomes also apparent after study of different cell types treated with PMA (Fig. 2). Only the promonocytic cell line U-937 and the myelomonocytic cell line HL-60 showed an increased expression of the antigen in response to PMA. This contrasts with the negative response of T, B and erythroblastoid cell lines.

As determined by immunoprecipitation analysis under reducing conditions, a broad band of 100-150 kDa was recognized by the mAb HC1/6 from cell surface radioiodinated U-937 cells after differentiation in the presence of PMA (Fig. 3, lane 2). The same relative molecular mass (M_r) was obtained under nonreducing conditions (lane 4) demonstrating the absence of interchain disulfide bonds. The CD11c/CD18 antigen complex was also immunoprecipitated for comparative purposes (lane 3).

3.2 Detection of the antigen recognized by the mAb HC1/6 in tissue MΦ

The antigen recognized by the mAb HC1/6 was visualized by immunoperoxidase staining of frozen tissue sections. Thus, MΦ from tonsil, lung and kidney were labeled by the mAb HC1/6 (Fig. 4). Scattered macrophages in the paracortical area (T cell zone) from tonsil (A), alveolar MΦ from lung (C) and mesangial MΦ from kidney glomeruli (B) were positively stained with mAb HC1/6. In addition, Kupffer cells from liver as well as MΦ from lymph node and thymus were labeled by mAb HC1/6, whereas microglial cells and dendritic epidermal cells were negative (data not shown). In most of these tissues, the presence of the HC1/6 antigen was evident in endothelial cells. Since the CD11c antigen is an excellent marker for tissue MΦ, we carried out a double staining with HC1/6 and anti-CD11c mAb (D). As shown by immunoperoxidase/alkaline phosphatase staining, the CD11c (brown) and HC1/6 (dark blue) antigens are co-expressed by the majority of MΦ. However, the HC1/6 antigen was expressed on vascular structures whereas the CD11c antigen was not.
Different pathologies characterized by the presence of an increased number of activated MΦ were also analyzed by staining of skin sections with the mAb HC1/6. In lepromatous leprosy and sarcoidosis, most of the infiltrating MΦ, as well as the epithelioid and giant cells present in the granulomas expressed the HCl/6 antigen (data not shown).

4 Discussion

The mAb HC1/6 described here recognizes a protein with a broad Mr, ranging from 100 to 150 kDa and which seems to be specific for the myeloid lineage. This antigen is highly expressed on peripheral blood Mo, tissue MΦ, platelets, endothelial cells and, upon differentiation, on the MΦ-like U-937 and HL-60 cells.

The expression of the antigen HC1/6 on Mo is not due to activation during the isolation protocol [37], since the same results were obtained when Mo were purified by the counter-current elutriation method [20]. In this sense, activation of Mo in the presence of IFN-γ down-regulates the expression of the antigen HC1/6 in contrast with an increased expression of MHC class II antigens. Although IFN-γ promotes differentiation of Mo into activated MΦ with cytotoxic activity and increased expression of MHC class II and FcR, certain myeloid differentiation antigens such as CD14 (Mo2) and 63D3 are down-regulated [36]. This is in agreement with the behavior of the antigen HC1/6 in the presence of IFN-γ but not observed in the presence of IFN-α.

Other myeloid antigens with a similar Mr as HC1/6 but different characteristics have been described [8, 9, 38, 39]. Thus, the antigens CD11b and CD11c are associated with a second β subunit (CD18) and are not present on peripheral platelets, as opposed to HCl/6. On the other hand, mAb of the CD13 cluster which are specific for a 150-kDa protein, differ from the antigen HC1/6 in their strong reactivity for bile canaliculi and granulocytes, the lack of staining of platelets and the distinct behavior upon stimulation with different agents (Fig. 1). Moreover, the CD31 antigen (sharp band at 130 kDa), although present on platelets, is highly expressed by peripheral granulocytes as well as by the T cell lines Jurkat and CEM as opposed to the antigen HC1/6 (broad band at 100–150 kDa).

A review of other unclustered antigens reveals that none is identical to the HCl/6 antigen. The β subunit of the VLA complex (110 kDa unreduced, 130 kDa reduced) which is identical to the gpIlla [30], is also present in platelets. However, this antigen is different from HCl/6 since: (a) the Mr of gpIlla changes between reducing or nonreducing conditions and (b) mAb directed against this antigen strongly react with the erythroblastoid cell line K-562 [41, 42]. Moreover, mAb MoU26, which recognizes a 130–140-kDa antigen [38] is distinct from the mAb HC1/6 due to the differential cellular reactivity since MoU26 strongly labels K-562 and does not react with Mo. Finally, the mAb CLB-HEC-75, which recognizes a 150-kDa antigen present on platelets and endothelial cells, is absent from Mo [43] and does not compete with the mAb HC1/6 for binding to PMA-treated U-937 cells (data not shown).

The mAb HC1/6 reacts with most of the vessels (venules, veins, small arteries and capillaries) and specially with high endothelial venules. Similarly, the ICAM-1 antigen and other members of the integrin superfamily are also expressed by endothelial cells [44]. Interestingly, the ICAM-1 antigen (ligand for LFA-1 antigen) is also up-regulated by PMA in U-937 and HL-60 cells [45, 46]. Since the antigens of the integrin family are involved in cell-matrix and cell-cell interactions, a similar function could be suggested for the antigen HC1/6. However, we have been unable to assign any functional role to the antigen HC1/6. In our hands, adhesion, migration antigen presentation and antibody-dependent cell-mediated cytotoxicity of Mo are unaffected by the presence of the mAb HC1/6. The fact that this antigen is not only present on Mo/MΦ but also in platelets, suggests that the function of the antigen HC1/6 is shared by both lineages.

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5 References


*Note added in proof:* While this manuscript was in press we found out that the mAb HC1/6 is reactive with transfectants of COS-7 cells expressing the CD31 antigen.

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