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Biochemical and antigenic characterization of CD45 polypeptides expressed on plasma membrane and internal granules of human neutrophils

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The expression of CD45 polypeptides, a phosphotyrosine phosphatase complex specific of leukocytes, has been investigated in both resting and activated neutrophils by using anti-CD45 monoclonal antibodies (MAb) which specifically recognize different polypeptide components of the CD45 molecular complex. Polypeptides of 180 and 130–150 kDa were equally precipitated by either a conventional CD45 MAb recognizing an antigenic determinant shared by the four CD45 glycoproteins (220, 205, 190 and 180 kDa) or by the anti-180 kDa UCHL1 MAb. These polypeptides were overexpressed on neutrophil plasma membranes after degranulatory stimulation. Conversely, neither the anti-220 kDa CD45R nor anti-220/205/190 kDa MAb reacted with CD45 molecules from resting or activated neutrophils. Furthermore, permeabilization analysis and comparative immunoprecipitation studies with different anti-CD45 MAb from fractions enriched in various neutrophil granules revealed that CD45 polypeptides (180 and 130–150 kDa) from internal granules are antigenic and biochemically identical to those expressed on plasma membrane.

Differentiation antigen; Polypeptide, CD45; Antigen specificity; Phosphotyrosine phosphatase; (Human neutrophil)

1. INTRODUCTION

CD45 (leukocyte common antigen, LCA, T200, Ly5) is a pan-leukocyte phosphotyrosine phosphatase glycoprotein complex showing a marked biochemical and antigenic heterogeneity [1–4]. This heterogeneity is due to the existence of cell-type-specific alternative splicing from a common precursor mRNA that generates several CD45 mature mRNAs coding for proteins with distinct amino acid content of the N-terminal regions [5–9]. Variable carbohydrate composition also contributes to the heterogeneity found within the CD45 molecular complex [10,11]. In human peripheral blood lymphocytes (PBL), CD45 is composed of four glycoproteins of 220, 205, 190 and 180 kDa, which are differentially expressed by distinct cell lineages [12]. We have previously characterized on PBL four different histological and biochemical specificities of CD45 defined by distinct anti-CD45 MAb [13]: (i) MAb recognizing the four CD45 glycoproteins (conventional pan-leukocyte CD45 specificity); (ii) MAb directed to the 220 kDa CD45 component (CD45R specificity); (iii) MAb recognizing the 220, 205 and 190 kDa CD45 components; and (iv) MAb specific for the 180 kDa CD45 polypeptide.

We have also recently reported the overexpression upon activation and the intracellular location of CD45 in human neutrophils [14], but in those studies the identity of CD45 components expressed on these cells was not established.

Here, the antigenic and biochemical nature of the distinct CD45 polypeptides expressed on

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Abbreviations: PBL, peripheral blood lymphocytes; PMA, phorbol myristate acetate; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine

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plasma membrane and internal granules of human neutrophils has been investigated.

2. MATERIALS AND METHODS

2.1. *Monoclonal antibodies*

The D3/9 and RP1/11 MAb are specific for conventional CD45 and CD45R, respectively [13,15]. MC5/2 MAb, directed against an epitope shared by the three CD45 polypeptides of largest size, displays identical reactivity to that of PD7/26/16 MAb, which originally defined such specificity [13,16]. The UCHL1 MAb recognizes an epitope only present on the 180 kDa CD45 polypeptide, and was kindly provided by Dr P.C.L. Beverley [13,17]. P3X63 IgG1 myeloma culture supernatant was used as negative control.

2.2. *Cells*

Cells were obtained from heparinized venous blood of normal volunteers. PBL were isolated by Ficoll Hypaque (Pharmacia, Uppsala) centrifugation. Neutrophils were isolated by subtraction of PBL as indicated above followed by sedimentation of erythrocytes in 1.3% (w/v) dextran at room temperature. Residual erythrocytes were removed by hypotonic lysis.

2.3. *Neutrophil activation*

Neutrophils were resuspended at 3×10^6 cells/ml in cold Hepes/glucose buffer (150 mM NaCl, 5 mM KOH, 10 mM Hepes, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5.5 mM glucose; pH 7.5) and incubated at 37°C with activating agent for 15 min. The Ca^{2+} ionophore A23187 was employed at 3×10^{-6} M, fMLP at 10^{-7} M, and PMA at 20 ng/ml. All reagents were from Sigma (St. Louis, MO). Cells held at 4°C or incubated at 37°C in the absence of any stimulus were run in parallel.

2.4. *FACS analysis*

Flow cytometry analysis was performed on an EPICS-C cytofluorometer (Coulter Scientific, Harpenden, England). Cells were incubated at 4°C with 100 μl MAb-containing culture supernatants, followed by washing and labeling with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse Ig. Fluorescence data were collected on a log scale and were converted to a linear scale for quantitative estimation. Specific linear fluorescence was obtained by subtracting background fluorescence produced by the negative control myeloma P3X63.

2.5. *Cell permeabilization*

Neutrophils were permeabilized as in [18]. Briefly, after cell fixation with 4% paraformaldehyde, 0.2% glutaraldehyde, 0.1 M sodium phosphate (pH 7.3) for 15 min at 4°C, and reduction in phosphate-buffered saline (PBS) containing 0.1% freshly prepared $NaBH_4$ for 10 min, neutrophils were incubated in PBS, 0.1% Triton X-100 for 15 min at 20°C. Then cells were washed twice with PBS containing 10% fetal calf serum, 10 mM Hepes, 5 mM EDTA and subjected to labeling with MAb and FITC-labeled goat anti-mouse Ig as described above.

2.6. *Subcellular fractionation of neutrophils*

Neutrophils were fractionated as described [19]. Briefly, postnuclear supernatant was layered onto a 15–40% (w/w) continuous sucrose gradient, and centrifuged at 76000 × *g* for 15 min at 4°C. Subsequently, fractions were collected by pumping 60% sucrose into the bottom. Each fraction was diluted with 50 mM Tris-HCl, 100 mM NaCl (pH 8.0) and centrifuged at 70000 × *g* for 90 min at 4°C. Pellets, representing the membranes of each fraction, were resuspended in 50 mM Tris-HCl (pH 7.5) and stored at −70°C. For radiolabeling experiments, membrane proteins were solubilized in borate saline buffer (0.01 M sodium borate, 0.14 M NaCl; pH 8.2) containing 0.2% Triton X-100.

2.7. *Radiolabeling, immunoprecipitation and electrophoresis*

Solubilized membrane proteins (50 μg) from the subcellular fractions or cell suspensions were radioiodinated in solution with chloroglycoluril (Iodo-Gen, Pierce, Rockford, IL). Cells were lysed in PBS (pH 7.4) containing 1% Triton X-100, 1% hemoglobin and 1 mM PMSF. For immunoprecipitation, equal amounts of input radioactivity of ^{125}I-labeled proteins from each subcellular fraction or cell lysates were mixed with 100 μl MAb-containing culture supernatants. To isolate immune complexes 100 μl of 187.1 anti-mouse κ-chain MAb followed by 30 μl protein A from *S. aureus* coupled to Sepharose (Pharmacia) were added. Immunoprecipitates were processed as in [20], and samples were subjected to SDS-7% PAGE and autoradiography.

3. RESULTS

To determine the antigenic and biochemical identity of CD45 proteins expressed on human neutrophils, immunoprecipitation experiments were carried out with ^{125}I-labeled neutrophil lysates using different anti-CD45 MAb (fig.1A). Only polypeptides of 180 and 130–150 kDa were precipitated from neutrophils by both conventional anti-CD45 D3/9 MAb and anti-180 kDa UCHL1 MAb (fig.1A, lanes 1,3, respectively). In contrast, no CD45 polypeptides were precipitated from neutrophils by MAb recognizing the 220/205/190 kDa CD45 components (MC5/2 MAb) or by the anti-CD45R MAb specific for the 220 kDa polypeptide (RP1/11 MAb) (fig.1A, lanes 2,4, respectively). Precipitations from ^{125}I-labeled PBL, where the four distinct biochemical patterns were established, were also included for comparison (fig.1B).

The reactivity of the different anti-CD45 MAb towards neutrophils and PBL was also studied by flow cytometry. Neutrophils were equally reactive with conventional CD45 D3/9 and anti-180 kDa UCHL1 MAb (fig.2A,C, respectively), whereas no staining was observed with the other two anti-CD45 MAb (MC5/2 and RP1/11 MAb) (fig.2B,D,

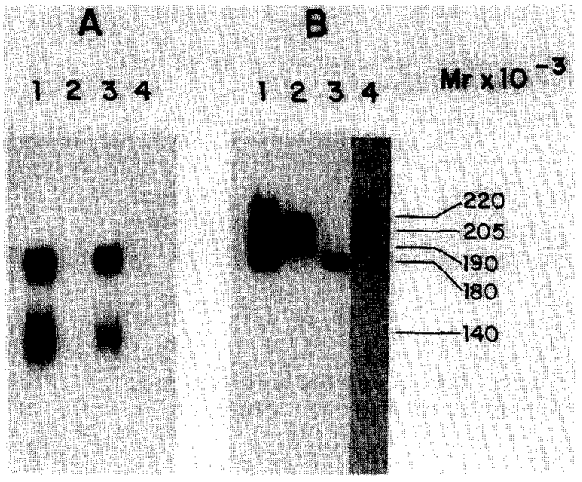

Fig.1. CD45 polypeptides precipitated by different anti-CD45 MAb from human neutrophils and PBL. Neutrophil (A) or PBL (B) ^{125}I-labeled lysates were precipitated by conventional anti-CD45 D3/9 MAb (lanes 1), anti-220/205/190 kDa MC5/2 MAb (lanes 2), anti-180 kDa UCHL1 MAb (lanes 3), and anti-CD45R RP1/11 MAb (lanes 4). Immune complexes were isolated as described in section 2, and reduced samples were subjected to SDS-7% PAGE and autoradiography.

respectively). However, four clearly distinct reactivity patterns were defined by these MAb on PBL (fig.2E–H).

Since we have previously reported that expression of CD45 on neutrophil plasma membranes can increase as a result of a stimulus-dependent translocation event, we also studied the antigenicity of CD45 molecules upregulated by this process. As shown in table 1, a significant increase in CD45 plasma membrane expression was observed when neutrophils were stimulated with different degranulatory agents, as detected by staining with either D3/9 or UCHL1 MAb. On the other hand, staining by MC5/2 or RP1/11 MAb remained negative after neutrophil activation. Furthermore, only reactivity with either D3/9 or UCHL1 MAb was detected when resting neutrophils were detergent-permeabilized to allow accessibility of CD45 internal molecules (table 1). Immunoprecipitation experiments on ^{125}I-labeled cell lysates did not reveal qualitative differences between CD45 polypeptides on plasma membranes from either resting or activated neutrophils (not shown).

To investigate further the identity of the intracellular proteins of CD45, subcellular fractionation studies were performed on resting neutrophils. Subcellular fractions were obtained by rate zonal sedimentation and fractionation was monitored by using the enzymatic markers alkaline phosphatase (plasma membrane), gelatinase (tertiary granules), lysozyme (specific granules) and β-glucuronidase (azurophilic granules) as described in [14]. Membrane proteins from the distinct subcellular fractions were radiolabeled and comparative immunoprecipitation experiments were carried out. As

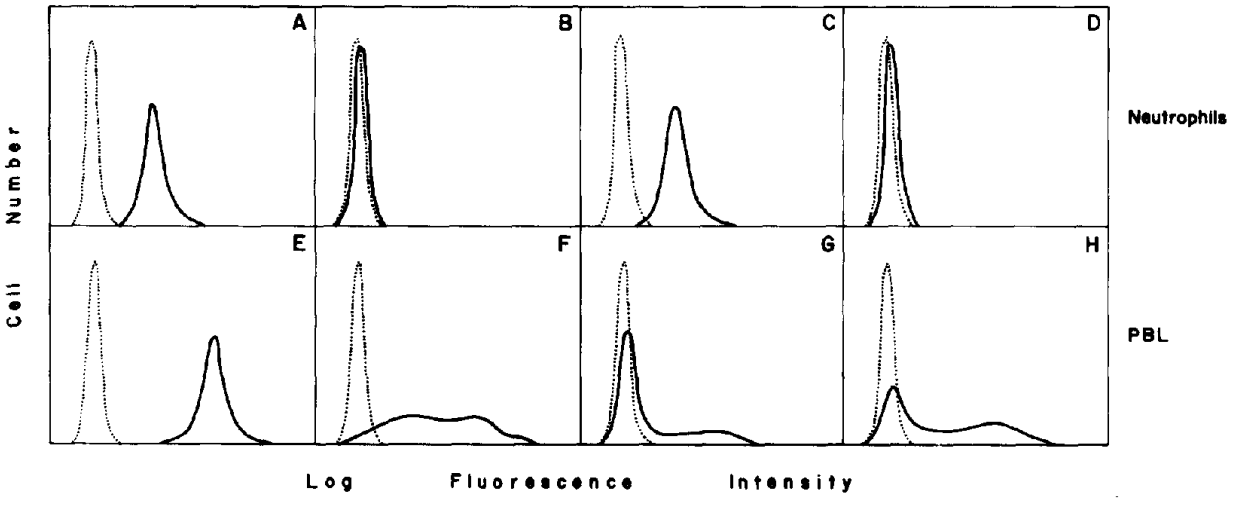

Fig.2. Immunofluorescence flow cytometry analysis of cells stained with different anti-CD45 MAb. Neutrophils (A–D) or PBL (E–H) were stained by D3/9 (A,E), MC5/2 (B,F), UCHL1 (C,G), or RP1/11 MAb (D,H) and analyzed as described in the text. Dotted lines correspond to staining with P3X63 negative control.

Table 1

Expression of CD45 biochemical specificities on human neutrophils upon incubation with different stimulating agents or upon permeabilization treatment

MAb	CD	Molecular mass (kDa)	Treatment					
			4°C	37°C	PMA	fMLP	Ca^{2+} ionophore	Permeabilization
D3/9	(CD45)	220,205,190,180	57 ± 9	93 ± 16	107 ± 19	117 ± 9	117 ± 18	125 ± 20
MC5/2		220,205,190	2 ± 1	1 ± 1	1 ± 1	1 ± 1	1 ± 1	2 ± 1
UCHL1		180	47 ± 10	59 ± 6	66 ± 7	86 ± 11	81 ± 14	78 ± 16
RP1/11	(CD45R)	220	1 ± 1	1 ± 1	2 ± 1	1 ± 1	1 ± 1	1 ± 1

Neutrophils were activated or permeabilized as described in the text. Data are expressed as means ± SE of specific linear fluorescence intensity mean values of four separate and independent experiments

shown in fig.3, CD45 polypeptides of 180 and 130–150 kDa, precipitated by either D3/9 or UCHL1 MAb (fig.3A,B, respectively), were present in large amounts in fractions 4–6, corresponding to the location of tertiary and specific granules. As observed, CD45 polypeptides from these fractions were enriched in the 130–150 kDa component with respect to those corresponding to plasma membranes (fig.3A,B, lanes 4–6 vs 2–3). By contrast, MC5/2 and RP1/11 MAb detected CD45 molecules in neither plasma membrane nor intracellular membrane fractions (fig.3C and not shown, respectively).

4. DISCUSSION

In this report, we have analyzed the internal and external expression of CD45 polypeptides on human neutrophils by using MAb that define four distinct CD45 biochemical reactivity patterns on PBL. We have found that only those MAb recognizing the four CD45 glycoproteins or the 180 kDa component were reactive with either plasma membrane or intracellular CD45 molecules from neutrophils, which were always resolved as polypeptides of 180 and 130–150 kDa. The polypeptide material of about 130–150 kDa

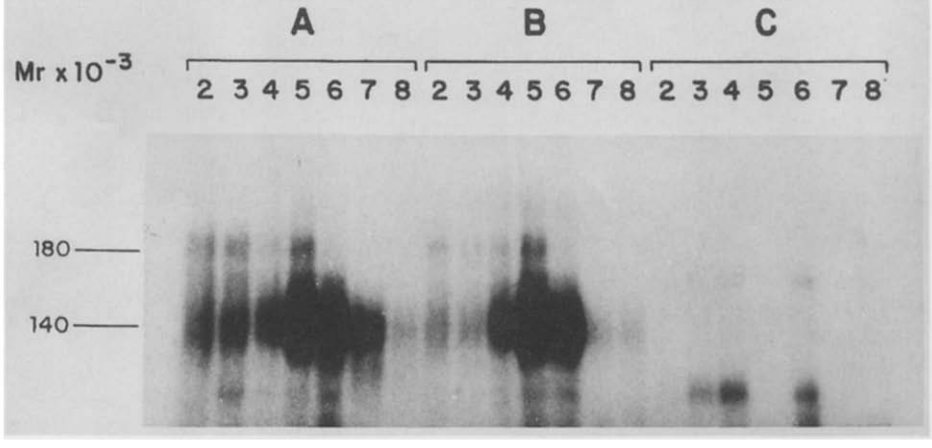

Fig.3. Immunoprecipitation by anti-CD45 MAb from ^{125}I-labeled lysates of membranes obtained from resting neutrophil subcellular fractions. Membranes from the distinct subcellular fractions, isolated as described in section 2, were solubilized and proteins were radioiodinated. Fractions (lanes): (2,3) plasma membrane; (4,5) tertiary granules; (5,6) specific granules; (8) azurophilic granules. Samples of each fraction were precipitated by D3/9 (A), UCHL1 (B), or MC5/2 MAb (C), and processed as described in the text.

probably was the result of proteolytic degradation of the 180 kDa protein. In precipitates with anti-CD45 MAb from neutrophil cell lysates stored for increasing periods of time, the 180 kDa polypeptide gradually disappeared whereas that of 130–150 kDa concomitantly increased (not shown). Alternatively, an autoproteolytic activity [21] or a granule-associated protease activity could also be responsible for the putative degradation during subcellular fractionation and storage. In this sense, our results showed that CD45 reactive material from internal granules was enriched in the 130–150 kDa component. Nevertheless, since we could not prevent this putative degradation with protease inhibitors (not shown), the possibility cannot be ruled out that the 130–150 kDa polypeptide is a normal constituent of human neutrophils.

Interestingly, the expression of the different CD45 proteins on resident or circulating leukocyte cell subsets appears to be related with the cellular maturation stage [13,22]. However, the physiological significance of this selective expression still remains unknown. It is important to note that human neutrophils only express the biochemical reactivity pattern of CD45 proteins defined by the UCHL1 MAb, that also reacts with monocytes and a subpopulation of T lymphocytes [13,17]. The 180 kDa CD45 specificity defined by this MAb is expressed by a helper inducer subset within the $CD4^+$ T cell population [17]. It has also bee reported that molecules recognized by UCHL1 MAb increase their expression on phytohemagglutinin activated mature and naive T cells, suggesting that this process would be associated with activation of post-thymic T lymphocytes [23–25]. The biochemical and functional relation between the 180 kDa polypeptide expressed by T lymphocytes and neutrophils remains to be determined.

Expression of CD45 proteins on neutrophil plasma membranes is up-regulated upon degranulatory stimulation [14]. Since a phosphotyrosine phosphatase activity has been recently demonstrated for CD45 molecules [4], the change in cellular location of this enzymatic activity could be crucial to the regulation of the neutrophil activation through local alterations in the phosphorylation state of phosphotyrosine proteins. As we show, the overexpression of CD45 does not affect the antigenic and biochemical nature of the CD45 neutrophil molecules. Such up-regulation is not dependent on protein synthesis and occurs rapidly, reaching a maximum increase about 15 min after addition of stimulus (unpublished), in a similar manner to that observed with other leukocyte glycoproteins implicated in cellular adhesion functions (leukocyte adhesion glycoprotein family LFA) [18,26]. These proteins mediate cellular aggregation and leukocyte adhesion to endothelial cells [27,28], but the existence of other different molecules has also been suggested to be implicated in such processes [29–31]. Interestingly, it has been recently reported that T lymphocytes from inflammatory infiltrates and those displaying a higher capacity to adhere to endothelium are mostly $UCHL1^+$ [32]. Furthermore, the interaction of a 180 kDa CD45 component with cytoskeletal elements has also been described [33]. Taken together, these observations indicate the possible validity of the speculation that CD45 180 kDa glycoproteins might be involved in regulation of leukocyte adhesive processes by selective dephosphorylation of different cellular substrates.

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