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CD11b IS A CALCIUM-DEPENDENT EPITOPE IN HUMAN NEUTROPHILS

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Summary: Human neutrophils expressing complement receptor 3 (CR3) were treated with various concentrations (0.04-10 mM) of Ca^{2+}/Mg^{2+}-chelating agent EDTA and the expression of CD11b, the CR3 α chain antigenic epitope, was examined using monoclonal antibodies and flow cytometry. EDTA caused a dose-dependent decrease in the reactivity of two anti-CD11b monoclonal antibodies, Leu15 and IOM1. The reduced expression of CD11b in EDTA-treated cells was partly restored by the addition of Ca^{2+} ions whereas the addition of Mg^{2+} ions had no effect on CD11b level. The expression of the CR3 ß chain epitope, CD18, was markedly decreased only by 10 mM EDTA. These results suggest that the CD11b epitope may be associated with the Ca^{2+}-binding domains of CR3 α chain and its recognition by antibodies depends on the presence of bound Ca^{2+}.

The human complement receptor 3 is a cell surface glycoprotein (CD11b/CD18) which plays a fundamental role in the adhesive reactions of human neutrophils. In addition to being receptor for C3bi opsonized particles (1,2), it binds unopsonized bacteria (3), yeast cell wall particles (4,5), fibrinogen and factor X (6), and promotes granulocyte cell-cell adhesion (7) as well as adhesion to endothelium (8). CR3 belongs to the same $ß_2$ integrin molecule subfamily as LFA-1 (CD11a/CD18) and p150,95 (CD11c/CD18). These three leukocyte integrin molecules are α/ß heterodimers composed of a common ß subunit noncovalently associated to unique α subunits (9). The complete amino acid sequences of the α subunits have been reported (10,11). Structural

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Abbreviations: CR3, complement receptor 3; mAb, monoclonal antibody; HBSS, Hanks' balanced salt solution; ID50, concentration required to induce 50 % inhibition.

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analyses have shown that the long extracytoplasmic NH_2-terminal region of CR3 α chain, like the α chains of LFA-1 and p150,95, contains four putative Ca^{2+}-binding domains (amino acid residues: 380-388, 449-457, 513-521, and 576-584) with homology to Ca^{2+}-binding sites found in calmodulin (12), paralbumin (13) and troponin C (14).

Several studies suggest that the metal-binding domains of CR3 regulate both the function and the structure of this receptor. It has been shown that CR3 binds ligands only in the presence of extracellular divalent cations (2,3). In addition, the occupancy of the metal-binding sites by Mg^{2+} or Mn^{2+} ions induces the expression of a novel antigenic epitope, which is not detected in the presence of bound Ca^{2+} (15,16).

The aim of this study was to investigate the role of divalent cations in monoclonal antibody (mAb) recognition of the antigenic epitope CD11b, an established cellular marker of CR3 expression.

Materials and Methods

Materials: EDTA was obtained from Fluka AG (Buchs, Switzerland). The phycoerythrin-conjugated anti-CD11b (Leu15) and purified anti-CD35 mAbs were purchased from Becton-Dickinson (Mountain View, CA, U.S.A). Purified anti-CD11b (IOM1) and anti-CD18 mAbs were from Immunotech S.A. (Marseille, France). The fluorescein isothiocyanate-conjugated goat anti-mouse Ig and phycoerythrin- and fluorescein isothiocyanate-labeled isotype control mAbs were from Becton-Dickinson. Calcium- and magnesium-salt free Hanks' balanced salt solution (HBSS) was prepared without phenol red and supplemented with 0.1 % gelatin. The total Ca^{2+} and Mg^{2+} concentrations of this HBSS were below 25 µM and 15 µM, respectively, as determined by atom absorption spectrometry.

Methods: Peripheral blood neutrophils were prepared as described (17). After isolation neutrophils (3×10^5/vial) were incubated for 20 min at 37 °C in 0.5 ml HBSS containing various concentrations of EDTA. Then the cells were pelleted (400 x g for 10 min at 4 °C), resuspended in 20 µl of cold HBSS containing mAbs in excess, as determined in saturation experiments, and the mixtures were incubated at 4 °C for 30 min. The cells were then washed with cold HBSS. Leukocytes, which were incubated with purified mAbs, were subsequently labeled with conjugated anti-mouse Ig for an additional 30 min. Controls included samples in which no first antibody was added and samples incubated with isotype-matched mAbs directed to an irrelevant antigen. The stained cells were finally suspended in Isoton III (balanced electrolyte solution, Coulter Electronics, Luton, England) and held on ice until examination by flow cytometry. A FACScan (Becton-Dickinson) was used for flow cytometry analysis of immunofluorescence.

Results

Treatment of isolated neutrophils with the Ca^{2+}/Mg^{2+}-chelating agent EDTA caused a striking, dose-dependent decrease in the expression of CD11b epitope (Fig 1). This fall in mAb recognition was apparent with both anti-CD11b mAbs used. The EDTA doses inducing 50 % inhibition in mAb binding were 1.8 and 3.6 mM for Leu15 and IOM1 mAbs, respectively. In contrast, EDTA at concentrations 0.04 to 4 mM did not markedly affect the expression of the CD18 surface antigen; only 10 mM EDTA was able to reduce the CD18 level more than 30 %. The chelating agent had no effect on the recognition of CD35 surface antigen (complement receptor 1), which does not contain Ca^{2+}/Mg^{2+}-binding sequences and thus has no requirements for divalent cations (3,18,19).

Cation dependency of the expression of the CD11b epitope was further studied by the addition of $CaCl_2$ or $MgCl_2$ to EDTA-treated cells. Interestingly, the addition of Ca^{2+} after 20 min incubation

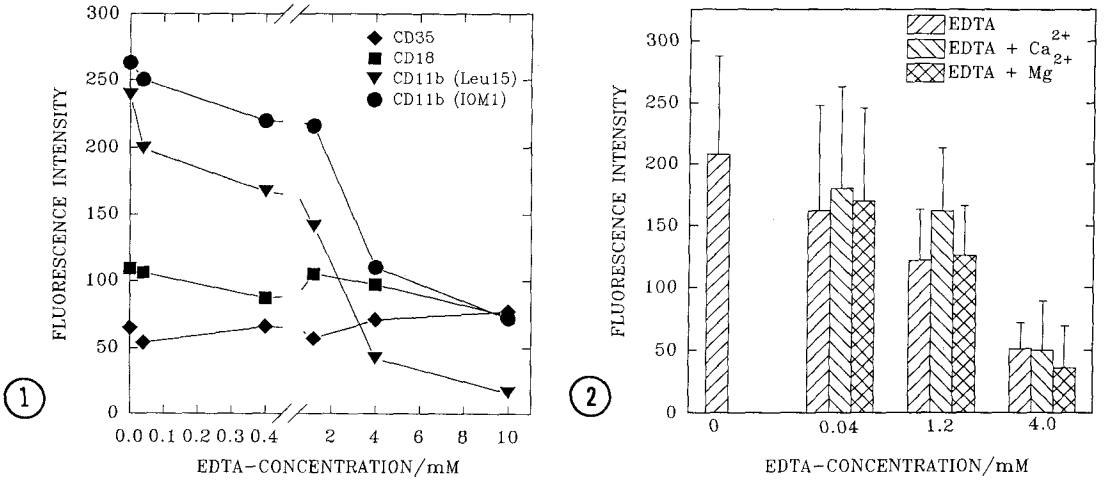

Figure 1. The effect of EDTA on the expression of neutrophil cell surface antigens. Isolated neutrophils were incubated for 20 min at 37 °C with various concentrations of EDTA and the antigen expression was determined using monoclonal antibodies and flow cytometry as described in Methods. Two different mAbs, Leu15 and IOM1, were used for quantitation of CD11b level. Results are presented as the mean fluorescence intensity on an arbitary scale from 0 to 10, 000. The mean of 5-7 separate experiments.

Figure 2. The effect of divalent cation restoration on anti-CD11b monoclonal antibody (Leu15) reactivity. Cells were pretreated with EDTA for 20 min at 37 °C to remove the bound cations. Subsequently 0.08, 2.4 and 8 mM Ca^{2+} or Mg^{2+} was added to reaction mixtures containing 0.04, 1.2 and 4 mM EDTA, respectively, and the cells were incubated for 20 min at 37 °C. The expression of CD11b was determined as described in Methods. Results are presented as the mean fluorescence intensity on an arbitary scale from 0 to 10,000. The mean and SD of 3 separate experiments.

with 0.04 or 1.2 mM EDTA could partially restore the expression of CD11b whereas there was no change in binding of the antibody after addition of Mg^{2+} (Fig 2). The expression of CD11b in cells treated with 4 mM EDTA could not be restored by addition of divalent cations. Even a 3-fold higher concentration of Ca^{2+} or Mg^{2+} (i.e. 12 mM) failed to increase the CD11b level (data not shown).

It has been shown that EDTA effciently blocks the mobilization of CR3 from intracellular stores (17,20,21). Therefore to exclude the possibility that the higher level of CD11b in control cells is due to the temperature-induced up-regulation of CR3 during the incubation at 37 °C, parallel incubations were performed at 4 °C. The mean (± SD) fluorescence intensity of Leu15 mAb labeled, cold-treated control cells was 227 ± 110 (n=4) which was almost identical to the results of control cells incubated at 37 °C.

Discussion

In this paper we have reported that the CR3 α chain monoclonal antibodies Leu15 and IOM1 define a divalent cation dependent epitope. This epitope is normally expressed on neutrophils but becomes absent when the divalent cations are removed from the metal-binding sites of the α subunit by the chelating agent EDTA. The structural features defined by Leu15 and IOM1 may therefore be part of one or more of the metal-binding sites or located at a closely associated domain. However, as EDTA-titration curves and the different ID50 concentrations for Leu15 and IOM1 indicate, these two mAbs probably do not recognize the very same structure on CD11b and, apparently, the Leu15 epitope is more sensitive to chelator induced changes.

Results from divalent cation restoration experiments suggest that Ca^{2+}, and not Mg^{2+}, is required for expression of CD11b epitope. This is consistent with the idea that the metal-binding sites are usually occupied by Ca^{2+} ions with relative high affinity even in Ca^{2+}- and Mg^{2+}-free medium (16). The finding that the antibody reactivity could be restored only partly suggests that the structural alterations imposed by the removal of Ca^{2+} are, at least to some extent, irreversible. One could speculate, for example, that the loss of one or two Ca^{2+} ions could be restored, but after the removal of several or all Ca^{2+} ions by high

concentration of EDTA, the conformation α chain adopts does no longer permit the Ca^{2+} binding.

Although the mAb recognition of CD18 was decreased after treatment of neutrophils with 10 mM EDTA, presumably because of the highly disordered conformation of the α subunit which alters the structure of the whole α/ß heterodimer, EDTA otherwise had only little effect on the expression of CD18 epitope. This and the unchanged expression of another cell surface receptor, CD35, argue against the possibility that EDTA unspecifically eg. by coating the cell surface inhibits the antibody reactivity.

In conclusion, the results presented here support the view that the metal-binding domains of CR3 α chain may refold in alternative conformations depending on the occupancy of these binding sites and also the nature of bound cation. Our findings also imply that the anti-CD11b mAbs Leu15 and IOM1 could be used to probe the binding of Ca^{2+} to CR3, analogously to mAb 24, which is used as a marker of the Mg^{2+} occupancy of leukocyte integrins (15,16).

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