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Activation of human neutrophil Mac-1 by anion substitution *

Elena Lomakina¹, Philip A. Knauf², Joanne B. Schultz², Foon-Yee Law², Matthew D. McGraw³, and Richard E. Waugh^{1,2,3}

¹Department of Pharmacology and Physiology, School of Medicine University of Rochester, Rochester, New York 14642

²Department of Biochemistry and Biophysics, School of Medicine University of Rochester, Rochester, New York 14642

³Department of Biomedical Engineering, School of Medicine University of Rochester, Rochester, New York 14642

Abstract

Substituting the medium chloride with glucuronate or glutamate causes a rapid, 10 to 30 –fold, increase in the binding of the monoclonal antibody, CBRM1/5, which recognizes the high-affinity conformation of the Mac-1 integrin. This change is reflected in functional adhesion assays that show increased adhesion to ICAM-1 coated beads. Blocking antibodies indicate that the increased adhesion is almost entirely due to Mac-1. The inhibitor NPPB (100μM) reduces Cl⁻ efflux into low Cl⁻ medium by 75%, and blocks increased CBRM1/5 binding after stimulation with fMLP or TNF-α, but has no effect on the anion substitution induced increase in CBRM1/5 binding or adhesion to immobilized ICAM-1. Thus, changes in *external* anion composition, not internal chloride or increases in Cl⁻ efflux, are responsible for Mac-1 activation. This effect is substantial. The percentage of Mac-1 in the high affinity state approaches 100% in glutamate and 50% in glucuronate, a far greater response than what is observed after stimulation with fMLP.

Keywords

adhesion molecules; cell surface molecules; adhesion; inflammation

Introduction

A critical step in the process by which neutrophils become selectively localized at sites of inflammation involves firm adhesion of receptors on the neutrophil to ligands on endothelial cells lining post-capillary venules, enabling neutrophils to migrate toward the site of inflammation rather than being swept away in the blood. Firm adhesion is triggered by conformational changes in neutrophil integrins (“activation”) that increase their affinity for binding to endothelial cell ligands such as intercellular adhesion molecule (ICAM-1) [1,2]. The importance of cations in integrin binding to ligands has long been recognized. Cations such as Mg²⁺ or Mn²⁺ can bind to the external portions of integrins [3–6], thereby causing conformational changes that are detected by binding of certain monoclonal antibodies, such as

Corresponding Author: Richard E. Waugh, Department of Biomedical Engineering, Robert B. Goergen Hall, University of Rochester, Rochester, NY 14627, Tel. 585 275 3768, Fax: 585 276 1999, e-mail: E-mail: Richard_Waugh@URMC.Rochester.edu.

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mAb24 [4,7] or CBRM1/5 [8,9], and are associated with increased affinity for ligands [6,10]. Crystal structures of integrins show several divalent cation binding sites, including the metal ion dependent adhesion site (MIDAS) in the I domain of the α subunits of many integrins, and the adjacent to MIDAS (ADMIDAS) site in the I-like domain of the β subunits [1].

While the importance of cations in regulating integrin conformation is widely recognized, the possible effects of anions on integrin conformation are relatively unexplored. The crystal structures of integrins in the ligand-bound forms show anionic amino acids, glutamate or aspartate, bound at the ligand binding site [9,11,12], yet the possibility that introduction of these amino acids into solution might affect integrin conformation has not been thoroughly tested. In the present report we demonstrate that inorganic anions in the cell environment can have a direct effect on integrin conformation and affinity. We use the activation-sensing monoclonal antibody CBRM1/5, which binds specifically to a portion of the I domain of the α_M chain [8,9], to detect effects of activators and changes in external anion composition on Mac-1 conformation. We also document corresponding changes in integrin affinity by measuring neutrophil adhesion to beads coated with the endothelial ligand, ICAM-1.

Materials and Methods

Reagents

Polymorph Cell Separation Medium was obtained from Accurate Chemical and Scientific Company. Bovine serum albumin (BSA), D-glucuronic acid sodium salt, L-glutamic acid monosodium salt hydrate, NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid), and fMLP were obtained from Sigma (St. Louis, MO). Human rTNF- α and ICAM-1 were purchased from R&D Systems (Minneapolis, MN).

Antibodies

Monoclonal antibodies ICRF44 (anti-human CD11b, IgG1), which binds to the α_M subunit and blocks Mac-1 ligand binding, R-PE-labeled and unlabeled mAb38 (anti-CD11a, IgG2a), which binds to the α_L subunit and blocks LFA-1 ligand binding, R-PE-labeled and unlabeled IB4 (anti-CD18, IgG2a), which binds to the β_2 subunit and blocks CD18 ligand binding, R-PE-labeled IgG2a isotype control and 15.2 (anti-human ICAM-1, IgG1) were purchased from Ancell (Bayport, MN). KIM127, which maps to I-EGF domain 2 in the β_2 leg, and AL57 Fab fragments, which report LFA-1 activation, were kindly provided by Dr. Minsoo Kim (University of Rochester, Rochester, NY). MAb 24 was a generous gift from Nancy Hogg (Cancer Research UK London Research Institute, London, UK). AL57 Fab fragments were labeled with AlexaFluor 488 antibody labeling kit (Invitrogen / Molecular Probes, Eugene, OR). FITC-labeled mAb CBRM1/5, which binds to the activated form of the I domain of human Mac-1 (CD11b/CD18) and FITC-labeled rat anti-mouse IgG were purchased from eBioscience (San Diego, CA). PE anti-human CD11b (Bear 1), PE-labeled IgG1 isotype control, and FITC-labeled IgG1 isotype control were obtained from Beckman Coulter Immunotech (Miami, FL). HUTS4 (IgG2b), which is specific to the active conformation of β_1 integrin, was purchased from Millipore (Temecula, CA). FITC-labeled MEM-101A (anti-human CD29, IgG1), which binds to the β_1 subunit, was purchased from Invitrogen Corporation (Carlsbad, CA). FITC-labeled anti-mouse IgG2b and FITC-labeled anti-mouse IgG1 were purchased from BioLegend (San Diego, CA). Quantum Simply Cellular Beads were purchased from Bangs Laboratories, Inc. (Fishers, IN).

Neutrophil isolation

Neutrophils were isolated from the whole blood using the Ficoll-Hypaque method [13]. After centrifugation the neutrophil-containing layer was collected, restored to normal osmolality by addition of an equal volume of balanced salt solution (BSS) (146 mM NaCl, 5 mM KCl, 5.5

mMD-glucose, 10 mM (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (HEPES)) that had been diluted 1:1 with distilled, deionized water, and then resuspended in 5 ml of BSS with 0.1 % BSA, pH 7.4. The cells were washed three times with this buffer, centrifuging for 10 minutes at 950 rpm (105 g) in an Eppendorf microcentrifuge. The cells were then resuspended at 5×10^6 cells per ml in BSS + 0.1% BSA buffer containing 1mM CaSO_4 and 1 mM MgSO_4 .

Low chloride experiments with CBRM1/5 antibody and NPPB

Neutrophils at 5×10^6 cells per ml were centrifuged in an Eppendorf microcentrifuge at 1500 rpm (165 g) for 5 minutes at room temperature and were resuspended to the original cell concentration in either BSS + 0.1% BSA with 1 mM Ca^{2+} and Mg^{2+} (as above), or in low chloride (low Cl^-) buffer (150 mM sodium glucuronate or 150 mM sodium glutamate or 150 mM sodium gluconate, 5.5 mM D-glucose, 10 mM Hepes, 1 mM CaSO_4 , and 1 mM MgSO_4 , pH 7.4) with 0.1% BSA, or at intermediate concentrations of glutamate or glucuronate substituted for Cl^- . Aliquots (100 μl) were taken immediately upon resuspension and at designated time points and placed on ice to cool. To assay the expression of epitope CBRM1/5, the cell suspensions were incubated for 1 hour at 4°C with FITC-labeled CBRM1/5 mAb (15 $\mu\text{g/ml}$). To assay total CD11b expression, cells were incubated for 45 minutes at 4°C with a saturating level (20 μl of the solution provided by the manufacturer) of PE anti-CD11b (Bear-1). Cells were washed three times in either low Cl^- buffer or BSS with 0.1% BSA at 4°C and fixed in 1% paraformaldehyde. Prior to flow cytometry, fixed cell samples were centrifuged once and resuspended in BSS.

In experiments with NPPB, 5×10^6 cells per ml were pretreated with the inhibitor for 5 minutes at 21°C in BSS + 0.02% BSA, followed by a 5 minutes centrifugation at 1500 rpm (165 g) at 21°C. Neutrophils were resuspended to original volume in low Cl^- buffer or BSS with 0.02 % BSA, with or without 100 μM NPPB. Cells were incubated for an additional 2 minutes at 37°C or room temperature and then 100 μl aliquots were placed on ice to cool prior to antibody labeling. For micropipette studies, 4% fetal bovine serum (FBS) was substituted for 0.02% BSA and after labeling cells were placed directly into the chamber on the microscope stage. In experiments with NPPB and stimulating agents, neutrophils were treated with 100 μM NPPB for 5 minutes followed by 15 minutes incubation at 37°C with 10 nM fMLP or 20 ng/ml $\text{TNF-}\alpha$.

Flow cytometry

Samples were analyzed in an Epics Elite (Coulter Instruments) flow cytometer or FACS Calibur (Becton Dickinson). Gates were set based on forward and side scatter to exclude red blood cells. 10,000 cells were analyzed for each sample. To correlate fluorescence intensity with the number of bound antibodies on cells or beads, the fluorescence signal was calibrated using Quantum Simply Cellular Beads (Bangs Laboratories, Inc., Fishers, IN) [13]. These beads provide a calibration of the Mean Fluorescent Intensity in terms of the number of antibodies bound to the surface. Thus, binding is expressed in terms of the antibody binding capacity, a quantitative measure of the number of antibody binding sites on the cell surface.

Chloride efflux in low Cl^- medium

Isolated neutrophils were washed twice in BSS, pH 7.4, with 0.1% BSA, at room temperature. The remaining red cells were lysed by resuspending the preparation in hypotonic buffer (14% v/v of Dulbecco's PBS without calcium or magnesium (Gibco) in distilled water) for 30 seconds, followed by the addition of 4X hypertonic PBS containing the appropriate amount of BSA to give a final isotonic solution with 0.1% BSA. Cells were then centrifuged, washed once in BSS containing 0.1% BSA, and resuspended to 2×10^7 cells per ml in the same buffer, except with the pH titrated to 7.4 at 37°C, for incubation with 2.5 $\mu\text{Ci/ml}$ $^{36}\text{Cl}^-$ for 1.5-2 hours

at 37°C. For each flux measurement, 0.9 ml of this suspension was washed twice in BSS containing 0.1% BSA at room temperature to eliminate excess isotope. Isotope-loaded cells were resuspended in 4.5 ml sodium glucuronate buffer (153 mM sodium glucuronate, 1.5 mM CaSO₄, 1 mM MgSO₄, 5.5 mM D-glucose, 10 mM HEPES, pH 7.4 at 21°C) containing 0.1% BSA at 37°C. If NPPB was present, only 0.01% BSA was used. At each predetermined time interval, 0.5 ml cell suspension was layered over 0.5 ml of a mixture of Silicone AR 200 fluid (Serva) and mineral oil (Sigma), with density 1.03 g/ml, in a 1.5 ml Eppendorf centrifuge tube. The sample was centrifuged immediately at 12,500 rpm (12,800 g) for 30 seconds in an Eppendorf microcentrifuge; sample time was recorded as the start of centrifugation. A 0.3 ml sample of the supernatant was taken, mixed with 0.4 ml SDS (0.1%), and added to 5 ml scintillation fluid to count for 10 minutes. Replicate 0.3 ml samples of the cell suspension were added directly to SDS and scintillation fluid to determine the total count per minute (cpm) in the suspension, y_{inf} . The rate constant for Cl⁻ efflux was calculated by fitting the cpm (y) at various times (t) to the equation $y = y_0 + (y_{inf} - y_0)(1 - \exp(-kt))$, where y_0 is the cpm at time zero. Values of y_0 and k were determined from a non-linear least squares fit using the program Origin (Microcal), with y_{inf} held constant at the measured value.

Coating beads for micropipette experiments

Recombinant soluble ICAM-1 (R&D Systems, Minneapolis, MN) was coupled covalently via tosyl linkage to paramagnetic M-450 Dynabeads (Dyna, Lake Success, NY) [6].

The density of ICAM-1 on ligand-coated beads was measured by flow cytometry. The beads were preincubated at 4°C overnight with FITC-conjugated mAb 15.2 against human ICAM-1. The beads coated with ethanolamine were used to detect background fluorescence. To correlate fluorescence intensity with the number of bound antibodies on cells or beads, the fluorescence signal was calibrated using Quantum Simply Cellular Beads. Beads used in these experiments had an ICAM-1 surface density of 250 or 140 sites/ μm^2 .

Micropipette Preparation and Technique

Micropipettes were made from glass capillary tubing as described previously [6]. The experiments were performed on the stage of an inverted microscope. Neutrophils were obtained in a drop of whole blood diluted into the appropriate buffer and placed in a dual-entry chamber on the microscope stage. One stationary pipette was used to hold a bead coated with ligand, and another pipette was used to hold the neutrophil and to manipulate the cell (Fig. 1). Neutrophils were individually selected based on their polymorphonuclear structure, which was clearly identifiable under light microscopy. The bead and the neutrophil were held in contact for 2 s, and then separated.

The contact and separation of each neutrophil - bead pair was recorded on videotape and analyzed subsequently to determine the fraction of contacts resulting in adhesion and to measure the contact area. Adhesion was noted as a deformation of the cell surface as the bead and cell were separated. The adhesion probability was calculated as the total number of adhesive events divided by total number of touches.

Results

Effect of Cl⁻ replacement on CBRM1/5 binding

Replacement of external Cl⁻ by glucuronate results in a marked increase in the activation state of Mac-1, as indicated by binding of the activation-epitope antibody CBRM1/5. The CBRM1/5 epitope is located in close proximity to the metal ion dependent adhesion site (MIDAS) in the Mac-1 I domain [9]. Prior studies have shown that increased binding of CBRM1/5 correlates with increased adhesion of Mac-1 to fibrinogen and binding of CBRM1/5 almost completely

blocks ligand binding [9]. Fig. 2A shows the changes in mean antibody binding capacity of neutrophils labeled with FITC-conjugated CBRM1/5 in the presence of the anion glucuronate as assessed by flow cytometry. In all cases the cell population appeared as a single Gaussian distribution around the mean fluorescent intensity. Antibody binding capacity was very low in BSS medium (open squares), but increased about 10-fold when cells were suspended in glucuronate medium (filled circles). The degree of increase was somewhat variable in cells from different donors, as indicated by the size of the standard error bars, but always substantial in comparison with the control binding in the 151 mM Cl⁻ BSS medium. The extent of activation was far larger than that seen with a classical neutrophil activator, fMLP (filled square at 30 minutes). The increase was rapidly reversed to near control levels when the cells were transferred from glucuronate medium back to chloride buffer (BSS) after 30 minutes incubation in glucuronate buffer (filled triangles). Both the increase (in low chloride) and decrease (in BSS) in CBRM1/5 binding were very rapid. To ensure that these apparently rapid changes were not due to slow conformational changes occurring over the course of the labeling process, cells were labeled first in glucuronate medium then washed into buffer with normal chloride content and CBRM1/5 binding levels were tested immediately. In this case, the labeling levels returned to control values.

In a separate series of experiments, we used the antibody KIM127, which maps to I-EGF domain 2 in the “leg” domain of the β_2 subunit and recognizes the high affinity state of all β_2 integrins, to verify that integrin conformational changes are induced by glucuronate. Substantial increases in KIM127 and mAb24 binding were observed in the presence of 60 mM glucuronate (data not shown). Using Fab fragments of the antibody AL57, which recognizes the high affinity state of LFA-1, we found no increase in LFA-1 activation under the same conditions. Thus, the increase in binding of KIM127 can be attributed totally to Mac-1 activation. Glucuronate does not appear to cause activation of β_1 integrins, as an antibody recognizing the active state of β_1 integrins (HUTS4) showed no differences in binding in the presence or absence of glucuronate.

Multiple neutrophil activating agents, including fMLP, TNF- α , and GM-CSF, are known to cause not only a change in β_2 integrin conformation but also an up-regulation of the amount of β_2 integrin on the cell surface [14]. The increase in β_2 integrin is mainly due to an increase in the amount of surface Mac-1, due to fusion of secretory vesicles with the plasma membrane [15,16]. To see whether or not such up-regulation could contribute to the increased CBRM1/5 binding observed, we measured the binding of a non-blocking anti-CD11b antibody (Bear-1) that binds to both the resting and activated forms of Mac-1. No significant changes in the amount of Bear-1 antibody binding capacity were seen upon exposure to glucuronate buffer or upon restoration of normal Cl⁻ levels (Fig. 2B). This is in sharp contrast to the large up-regulation of Mac-1 caused by fMLP (filled square). Thus, the changes in CBRM1/5 binding seen in Fig. 2A must represent changes in conformation of Mac-1 molecules already present at the cell surface.

Comparison of the number of CBRM1/5 binding sites and the number of Bear-1 binding sites (Fig. 3A) reveals that replacement of Cl⁻ with glucuronate (filled circles) causes a change in conformation of a very large portion of the Mac-1 molecules, approximately 40-50% as measured by the antibody binding capacities of the two antibodies. This contrasts with the effects of fMLP (filled square at 30 minutes), where the ratio of activated (CBRM1/5 binding) to total (Bear-1 binding) Mac-1 is only slightly increased relative to the control (open squares). Similar results were obtained at 21°C (Fig. 3B). Assuming that each antibody bonds to one copy of Mac-1, the number of copies of Mac-1 on the cell surface increases from approximately 100,000 in resting cells to approximately 300,000 in cells treated with fMLP. In contrast, the number of activated forms increases from a baseline level of approximately 1,500 activated forms per cell to approximately 5,000 copies per cell in fMLP, but to approximately 50,000

sites per cell in the presence of glucuronate. This emphasizes the high degree of effectiveness of anions in altering integrin conformation. Even though fMLP causes a dramatic increase in the number of Mac-1 molecules expressed on the surface, the number of molecules that bind CBRM1/5 after fMLP exposure is substantially lower than that measured in the presence of glucuronate, even though glucuronate causes little or no increase in the number of Mac-1 molecules on the cell surface.

To further investigate the effect of Cl^- substitution on the activation of Mac-1, the anions *glutamate* and *gluconate* were used in place of Cl^- . As in the case of glucuronate, CBRM1/5 antibody binding was used to estimate the amount of active Mac-1, and Bear-1 antibody was used to estimate the total amount of Mac-1 on the cell surface. Both substitutes caused an increase in CBRM1/5 binding, as shown by the ratio of CBRM1/5 to Bear-1 in Figure 4. Gluconate resulted in increases similar to those of glucuronate, but in glutamate the increase was even more substantial. As was the case for glucuronate, neither of these anions caused significant increases in the number of Mac-1 molecules expressed on the surface, even though the number of activated forms increased substantially. The occurrence of a conformational change was confirmed by observations of substantial increases in binding of KIM127, which recognizes all active β_2 integrin forms (data not shown). Unlike glucuronate, glutamate caused mild activation of LFA-1 as assessed by AL57 binding, although it was approximately 10 times less than that observed for Mac-1. As in the case with glucuronate, none of the β_1 integrins were activated in the presence of glutamate, as indicated by HUST4 binding.

Concentration dependence of chloride anion substitute effects on CBRM1/5 binding

To evaluate concentration dependence of the effect of chloride replacement on Mac-1 activation, varying ratios of Na glucuronate / Na chloride, as well as Na glutamate / Na chloride were tested. As shown in Figure 5, increasing the amount of anion substitute and decreasing the amount of chloride in the external medium caused a concomitant increase in amount of Mac-1 in the active state, as reflected by CBRM1/5 binding. The data fit very well to a Michaelis-Menten dependence, a relationship describing a first order binding interaction. The residual variance (r^2) of the fit was 0.97 in glutamate and 0.89 in glucuronate, providing additional evidence for external ion binding as the mechanism underlying the change in integrin conformation. The concentration for half saturation of the effect was approximately 25 mM for both glucuronate and glutamate.

Ruling out calcium effects

We explored the possibility that the chelation of calcium by glutamate or glucuronate might be contributing to the increase in Mac-1 activation. This possibility was contraindicated by the observation that removal of calcium in the absence of glutamate or glucuronate had no effect on CBRM1/5 binding. Furthermore measurements of calcium concentrations using a Ca^{2+} electrode indicated that calcium concentrations remained in the mM range for all buffers tested. For calcium concentrations ranging from 1 mM to zero (no calcium added and the presence of 0.5 mM EGTA) the same levels of CBRM1/5 binding were observed as for control conditions (in BSS) (data not shown).

Evaluation of Cl^- efflux and intracellular Cl^- as mediators of integrin activation

Prior studies have suggested that the effects of low Cl^- media on integrin conformation were due to changes in intracellular Cl^- concentration [17]. To evaluate the likelihood of this hypothesis for the increases in CBRM1/5 binding observed here, we measured Cl^- efflux from neutrophils suspended in low Cl^- glucuronate by loading the cells with radioactive $^{36}\text{Cl}^-$ and observing the rate of appearance of $^{36}\text{Cl}^-$ in the medium (Fig. 6). The data for neutrophils in low Cl^- medium (open circles) fit well to a single exponential (solid line) with an average rate constant ($n=6$) corresponding to loss of 3.1% of intracellular Cl^- per minute. Most of the

increase in CBRM1/5 was seen by 2 minutes (Fig. 2A), at which time intracellular Cl^- would have decreased by only about 6%. Keep in mind however that during the labeling time (45 minutes at $2-4^\circ\text{C}$) cells remained suspended in glucuronate buffer, and could have continued to lose Cl^- , albeit at a slower rate.

When we exposed $^{36}\text{Cl}^-$ -loaded neutrophils to 100 μM NPPB, a well-known inhibitor of Cl^- channels [18], the Cl^- efflux was greatly inhibited (Fig. 6, solid circles and dashed line). This concentration caused an average of 75% inhibition of Cl^- efflux. If the decrease in internal Cl^- concentration or the magnitude of the Cl^- efflux in low Cl^- medium were responsible for the increased CBRM1/5 binding, then inhibition of these processes by NPPB should reduce the binding. Contrary to this prediction, we observed no decrease in the CBRM1/5 binding in low Cl^- media with NPPB (Fig. 7), indicating that the increase in CBRM1/5 binding is caused by replacement of extracellular Cl^- with glucuronate, and not by changes in intracellular Cl^- concentration or Cl^- efflux rate.

To further test whether the increase in CBRM1/5 binding in the low chloride buffers was due changes in intracellular chloride or simply to external anion binding, glutamate or glucuronate was simply added to the buffer to make it hypertonic. When 30mM or 60mM glutamate was added to BSS buffer, the cells exhibited 7-10 times higher CBRM1/5 binding compared to addition of 30mM or 60mM NaCl added to the same buffer (Fig. 8). In case of glucuronate addition (30mM or 60mM), CBRM1/5 binding also increased significantly. Note that in the hypertonic buffers, the intracellular chloride concentration should increase because of cell dehydration, whereas in the replacement experiments, in which the external chloride is reduced, the internal chloride concentration is expected to decrease slightly because of chloride efflux. Yet in all cases, the increase in CBRM1/5 binding correlated with the concentration of glutamate or glucuronate in the external medium, regardless of the expected changes in intracellular chloride concentration.

Chloride channel involvement in activator-induced conformational changes

In a previous study, evidence was presented that chloride channel inhibitors could prevent integrin conformational changes in response to inflammatory agonists [17]. In that study, $\text{TNF-}\alpha$ (10 ng/ml) caused a 3-fold increase in mAb24 binding that was prevented by the chloride channel inhibitor ethacrynic acid. We performed similar experiments with both $\text{TNF-}\alpha$ and fMLP as activators, using NPPB to inhibit Cl^- channels. In a single experiment, 20 ng/ml $\text{TNF-}\alpha$ caused CBRM1/5 binding to increase by 105% above the corresponding control. The increase was reduced to 30% above control with 100 μM NPPB. Similarly, fMLP at 10 nM caused an increase in CBRM1/5 binding that was almost completely prevented by NPPB (Fig. 9). Total Mac-1 surface expression, as measured by binding of the Bear-1 mAb, was also increased after treatment with fMLP (Fig. 9), and this increase too was inhibited by NPPB. The strong inhibitory effect of NPPB in these experiments is in sharp contrast to the lack of effect of NPPB on the anion substitution stimulated increase. In contrast to the lack of inhibition by NPPB of the external anion-induced integrin activation, the inhibition by NPPB of the responses to fMLP and $\text{TNF-}\alpha$ is consistent with the hypothesis that the effects of these agents on CBRM1/5 binding *are* related to increased Cl^- efflux or a decrease in internal Cl^- concentration, as originally postulated [17]. However, it cannot be concluded that Cl^- efflux or a decrease in internal Cl^- concentration alone would be sufficient to *cause* integrin activation.

Effect of low Cl^- medium on neutrophil adhesion to ICAM-1 coated beads

Finally, we demonstrated that inorganic anions increase integrin-mediated neutrophils adhesion by measuring changes in the probability of adhesion of neutrophils to beads coated with the endothelial cell ligand ICAM-1 (Fig. 10). This approach was developed by Zhu and colleagues [19] and has been used by us to evaluate 2-D binding kinetics of neutrophils to ICAM-1 in the

presence of Mg^{2+} /EGTA [6]. Purified neutrophils in glucuronate and glutamate exhibited a much higher adhesion probability ($\sim 60\%$), compared with neutrophils in BSS buffer (20%). This increase is comparable to what we have demonstrated previously in the presence of Mg^{2+} /EGTA [6]. The increase in adhesion in the presence of glutamate or glucuronate was attributable almost entirely to Mac-1 binding. Treatment with antibodies that specifically block Mac-1 binding reduced adhesion probability to the control level. CBRM1/5 and ICRF44, both Mac-1 blocking antibodies, were equally effective at blocking the increase in adhesion. The anti- β_2 integrin blocking antibody IB4, which should block adhesion to both LFA-1 and Mac-1, caused no further reduction in adhesion. In contrast to the effects of Mac-1 blocking antibodies, antibodies that block LFA-1 interactions showed no effect on binding in glucuronate, although a partial reduction in binding in the presence of glutamate was observed. Addition of NPPB had no effect on adhesion in either glutamate or glucuronate buffer. These data show that the glutamate or glucuronate induced changes in Mac-1 conformation detected by activation epitope antibodies had functional consequences in that the cell's ability to form adhesive bonds with ICAM-1 was increased significantly.

Discussion

The effect of divalent cations on integrin conformation and affinity is well known [5-7,20], but the results reported here show that specific anions can also have a substantial effect on integrin conformation and affinity. Perhaps most surprising is the high level of integrin activation in the presence of moderate concentrations of these anions. The percentage of surface-expressed Mac-1 in the high affinity state, as indicated by CBRM1/5 binding was approximately 50% in the presence of glucuronate or gluconate and approached 100% in the presence of glutamate. This percentage of activated forms is substantially higher than what is observed in response to the bacterial agonist fMLP or to TNF- α . Also, in contrast to the effects of fMLP or TNF- α , the increase in the number of activated integrins is not accompanied by an increase in the number of surface-expressed integrins on cells, suggesting that the effect is principally an externally mediated effect and is not accompanied by general activation of the cell. This is further supported by the dependence of integrin activation on anion concentration, which is consistent with a simple first order binding interaction with a dissociation constant of approximately 25 mM.

Substitution of glucuronate or glutamate for Cl^- maintains ionic strength constant. This argues against the possibility that the increase in CBRM1/5 binding is likely to be related to nonspecific alterations of charge-charge interactions in the integrin heterodimer, and further supports our conclusion that there are specific effects on protein conformation related to external anion binding. In the case where glutamate or glucuronate was added in addition to physiological concentrations of Cl^- , smaller increases in CBRM1/5 binding were observed. This is consistent with other observations [21] that hypertonic conditions can actually decrease integrin activation levels. That glutamate or glucuronate can increase CBRM1/5 binding without reduction in Cl^- argues strongly that the effect is due to an association of these anions with the extracellular domain of the integrin, although the possibility that the binding site is on an adjacent protein that interacts with the integrin cannot be ruled out.

Sites for binding of anions to the extracellular domains of integrins are not so well characterized as those for cations, but two prior lines of investigation suggest mechanisms for the different effects of glutamate vs. gluconate or glucuronate. One of these is the identification of a glutamic acid residue in the binding site of ICAM-1 that coordinates with the metal ion in the metal ion binding site (MIDAS) of integrins [22]. This binding replaces other negatively charged residues that coordinate in the MIDAS region of the integrin in its closed, low-affinity conformation [23-26]. It seems plausible that the presence of glutamate in solution at sufficient concentrations would also induce a conformational change from the closed (low-affinity) to the open (high

affinity) conformation. It is less clear whether gluconate or glucuronate might act by the same mechanism or whether they may act by interacting with a different portion of the molecule. Vetvicka [27] demonstrated that soluble β -glucan polysaccharide binding to the lectin site of neutrophil CD11b/CD18 generates a primed state of the integrin. Polysaccharide priming resulted in a magnesium dependent conformational change of the I domain that exposed the CBRM1/5 epitope. Testing of β -glucan binding to recombinant Mac-1 fragments indicated that the polysaccharide binding site is distinct from and does not include MIDAS. Thus it seems likely that the different levels of response we have observed in integrin activation by these different anions may be the result of their action through distinctly different mechanisms.

The involvement of chloride in the regulation of integrin-mediated adhesion was first indicated in a study by Vedder and Harlan [28] in which it was shown that the anion exchange inhibitor DIDS prevented upregulation of CD18 expression and increased neutrophil aggregation after stimulation with inflammatory agents. This was consistent with the earlier reports that DIDS blocked granule release in neutrophils, suggesting that chloride exchange plays a role in fusion of internal vesicles with the plasma membrane after stimulus. Their observation that DIDS did not affect neutrophil adhesion to endothelium after inflammatory stimulus was surprising, but could be attributed to the likelihood that integrin activation and expression are regulated by different mechanisms, or simply that other adhesive ligand pairs may have mediated the endothelial attachment. In a more recent study, Menegazzi et al [17] demonstrated that TNF- α caused an increase in binding of both CBRM1/5 and mAb24, as well as an increased rate of Cl^- efflux. Two inhibitors of Cl^- efflux, ethacrynic acid and MK447/A, inhibited the increase in mAb24 binding. Our evidence that another anion transport inhibitor, NPPB, blocks the TNF- α or fMLP induced increase in CBRM1/5 binding supports the concept that Cl^- efflux is required for the change in α_M I domain conformation in response to inflammatory agonists. However, our results do not support the hypothesis that a decrease in intracellular Cl^- alone is sufficient to induce integrin activation because NPPB does not inhibit either an increase in CBRM 1/5 or an increase in ICAM-1 adhesion in the presence of either glutamate or glucuronate. (The combination of chloride flux inhibition and low external chloride was not tested in the earlier study.) Rather, our findings provide the first evidence for a conformational change in Mac-1 that is caused by the *external* anion composition. It seems unlikely that the change in integrin conformation is due to the lack of chloride, per se. Glutamate produces much larger changes in CBRM1/5 than glucuronate at the same reductions in chloride concentration, and these effects persist when these anions are added *in addition* to physiological concentrations of chloride. All of this points again to the conclusion that a direct interaction between Mac-1 and either glutamate or glucuronate is responsible for the conformational change in the protein.

The large increase in the fraction of Mac-1 molecules displaying the activated conformation detected by CBRM1/5 leads to a nearly 3-fold increase in the probability of adhesion to ICAM-1 coated beads in both buffers tested. The fact that nearly the entire increase in adhesion was blocked by Mac-1 specific antibodies (CBRM1/5 or ICRF44) indicates that the increased adhesiveness was almost all related to Mac-1 interactions with ICAM-1. This agrees with earlier studies that demonstrated a strong correlation between CBRM1/5 binding and Mac-1 binding affinity for one of its ligands [8]. That Mac-1 is the principal adhesive ligand in these studies is further supported by observations that an antibody blocking LFA-1 interactions did not reduce adhesion in glucuronate, and reduced it only partially in the presence of glutamate. Interestingly, the dramatic involvement of Mac-1 in anion-induced adhesion to ICAM-1 is opposite to what was found in a previous study, in which we demonstrated that in Mg^{2+} /EGTA the preponderance of adhesion to ICAM-1 was mediated by LFA-1, and that Mac-1 played a minor role, if any. The apparent lack of involvement of LFA-1 in the glucuronate-induced adhesion raises the possibility that transformation of this integrin from its low to high affinity state must occur by mechanisms that are less sensitive to presence of glutamate and

glucuronate, and which may be substantially different from the conformational changes that have been characterized for Mac-1.

Although the concentrations of these anions used in the present experiments are well above what is typically encountered physiologically, small concentrations of anion are required to induce the same percentage change in CBRM1/5 binding as is observed for bacterial peptides and other neutrophil agonists. Typically, fMLP results in CBRM1/5 binding to approximately 10% of the Mac-1 on the cell surface [8]. Based on the concentration dependence measured in the present study, a glutamate concentration of approximately 3.0 mM is sufficient to cause the same percentage change. Thus, although the required concentrations are greater, the resulting effects of these anions are akin to conformational changes produced by divalent cation substitution, and represent a valuable alternative for altering Mac-1 affinity and neutrophil adhesion.

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Fig. 1.

Interaction between a neutrophil and an ICAM-1 coated bead during the adhesion experiment: **A.** An ICAM-1 coated bead (4.5 μm diameter) is held in the left pipette and the neutrophil is held in the right pipette. **B.** The contact between the beads and the cell. Adhesion events are scored when there is a small deflection of the cell surface as the cell and bead are separated. The adhesion probability is calculated as the number of adhesive events over the total number of touches.

A.

B.

Fig. 2.

Effect of glucuronate on Mac 1 expression and activation. Neutrophils (5×10^6 cells/ml) were either maintained in BSS (opened squares) or resuspended at time zero in glucuronate buffer (filled circles). 100 μ l aliquots were taken at the indicated times and placed on ice. At 30 minutes a portion of cells in glucuronate buffer was centrifuged, resuspended in BSS, and returned to the 37°C water bath (filled triangles). Neutrophils were also stimulated with 10 nM fMLP for 30 minutes in BSS (filled square). Antibody binding capacity (ABC) was obtained using Quantum Simply Cellular Beads. Each point represents the mean of three experiments, each with duplicate determinations, with cells from different donors, except for the fMLP data, where the mean of two experiments is shown. Experiments were performed at 37°C. Error bars indicate standard error of the mean (s.e.m.) or, for the fMLP data, range of the two values. **A.** Antibody binding capacity for the Mac-1 activation epitope, recognized by CBRM1/5 antibody

binding. **B.** Antibody binding capacity for the total Mac-1 surface expression, recognized by Bear-1 antibody binding.

A.

B.

Fig. 3.

Effect of glucuronate on the fraction of Mac-1 in the activated (CBRM1/5 binding) state at **A.** 37°C and **B.** 21°C. The fraction of Mac-1 displaying the CBRM1/5 activation epitope was determined by dividing the number of antibody binding sites for CBRM1/5 by the number of binding sites for Bear-1. Mean fluorescence was converted to antibody binding capacity by calibrating with Quantum Simply Cellular Beads. BSS – opened squares; Na glucuronate buffer – filled circles; cells in Na glucuronate returned to BSS after 30 minutes – filled triangles; 10 nM fMLP stimulated cells – filled square. Means from 3 experiments at 37°C (except for the fMLP data, which represents 2 experiments), together with s.e.m. or range (for fMLP data) are shown in **A.**; **B** represents a single experiment with duplicate determinations.

Fig. 4.

Effect of different anions on the fraction of Mac-1 in the active state. The fraction of active Mac-1 was determined as described in Fig. 2. Na glutamate – filled rhombuses; Na glucuronate – filled circles; Na gluconate – filled stars; BSS – opened squares. Experiments were performed at 21°C. For glutamate, fractions greater than 1.0 reflect measurement uncertainty, either in underestimating the total integrin on the cell surface (obtained from Bear-1 binding) or overestimating activated forms (CBRM1/5 binding).

A.

B.

Fig. 5.

Effect of varying chloride substitute concentration on the expression of Mac-1 activation epitope, recognized by CBRM 1/5 binding. The fit is done using the form of the Michaelis-Menten equation: $f = f_{max}(x/(K_m + x))$, where f_{max} is the fraction of integrins in the high affinity state at saturating concentrations of anion and K_m is the Michaelis-Menten constant, which corresponds to the apparent K_d of the binding reaction. The ratio was calculated based on CBRM1/5 binding measured at each concentration, and an estimate of the total number of integrins on the surface based on the data in Figure 3. **A.** Chloride was substituted with glucuronate (open circles), glutamate (closed squares) is shown as a reference: $f_{max} = 0.55 \pm 0.04$, $K_m = 24.7 \pm 7.2$ mM, $R^2 = 0.894$. **B.** Chloride was substituted with glutamate (closed

squares), glucuronate (open circles), is shown as a reference: $f_{max}=1.12 \pm 0.04$, $K_m=25.9 \pm 4.3$ mM, $R^2=0.973$.

Fig. 6.

NPPB inhibition of Cl^- efflux in low Cl^- medium. Net chloride efflux from $^{36}\text{Cl}^-$ loaded cells into Na glucuronate buffer at 37°C was measured as described in Materials and Methods. The cpm of $^{36}\text{Cl}^-$ are plotted as a function of time after resuspension in the glucuronate medium. The plot shows typical data for control efflux (open circles) and efflux into medium with $100\ \mu\text{M}$ NPPB (filled circles). In 3 experiments, with blood from 3 different donors, the average control rate constant was 0.031 (SE 0.005, $n=6$) and the average with NPPB was 0.0077 (s.e.m. 0.0020, $n=5$). Thus, on average $100\ \mu\text{M}$ NPPB reduced Cl^- efflux to 25% of the control rate.

Fig. 7.

Effect of NPPB on CBRM1/5 surface expression in Na glucuronate and Na glutamate buffer. Neutrophils (5×10^6 cells/ml) were incubated in BSS with or without NPPB. Antibody binding capacity (ABC) was obtained using Quantum Simply Cellular Beads as described in Materials and Methods. For Na glucuronate each column shows the mean of four experiments with neutrophils from four different donors (each with duplicate samples). The error bars represent s.e.m. For Na glutamate, each bar represents the mean of two experiments, each with two replicates.

Fig. 8.

Effect of external anions on neutrophil Mac-1 activation epitope expression, recognized by CBRM1/5 antibody binding. Each bar represents the mean of three experiments, each with duplicate determinations, with cells from different donors (except 37.5 mM glutamate where $n=1$). Experiments were performed at room temperature. The error bars represent s.e.m. Cases shown as +30mM or +60 mM were hypertonic. The 37.5 mM buffers were obtained by replacement of chloride and were isotonic. Applying ANOVA and Duncan's multiple range test ($\alpha = 0.05$), we find that differences among the four control BSS buffers (leftmost columns) were not statistically significant, and the differences among the three glucuronate buffers (center columns) were also not statistically significant. However, all glucuronate and glutamate buffers were significantly different from controls, the two glutamate concentrations (30 mM and 60 mM) were different from each other and different from all glucuronate-containing buffers.

Fig. 9.

NPPB inhibition of fMLP-induced CBRM1/5 epitope expression and total Mac-1 expression. Neutrophils (5×10^6 cells/ml) were treated with or without 100 μ M NPPB for 5 minutes at 37°C. Cells were then stimulated without (control) or with 10 nM fMLP for 15 minutes at 37°C. 100 μ l aliquots were immediately placed in cold microcentrifuge tubes on ice for antibody labeling. Quantum Simply Cellular Beads were used to determine antibody binding capacity. Each column represents the mean of two experiments (with duplicate determinations in each) and the error bars indicate the range for the three experiments.

Fig. 10.

Effect of chloride substitutes on neutrophil adhesion to ICAM-1. Experiments were performed in BSS, in low Cl^- medium (glucuronate or glutamate), or in low Cl^- medium in the presence of mAb38 (LFA-1 blocking antibody), ICRF44 (Mac-1 blocking antibody), IB4 (β_2 blocking antibody) or 100 μM NPPB as indicated. Each cell-bead pair was contacted 25 times for 2 seconds each time. Adhesion was detected as described in Materials and Methods, and adhesion probability was calculated from the number of adhesive events divided by the number of contacts. Each column represents the mean value from experiments on 4-6 cells from each of 2-4 different donors. Error bars represent s.e.m.