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Differential modulation of annexin I binding sites on monocytes and neutrophils

H. S. Euzger,[1] R. J. Flower,[2] N. J. Goulding[1] and M. Perretti[2,CA]

Department of Biochemical Pharmacology, [1]Arthritis Research Unit, and [2]The William Harvey Research Institute, Charterhouse Square, London EC1M 6BQ, UK

[CA]Corresponding Author
Tel: (+44) 171 982 6065
Fax: (+44) 171 982 6076
Email: m.perretti@qmw.ac.uk

Specific binding sites for the anti-inflammatory protein annexin I have been detected on the surface of human monocytes and polymorphonuclear leukocytes (PMN). These binding sites are proteinaceous in nature and are sensitive to cleavage by the proteolytic enzymes trypsin, collagenase, elastase and cathepsin G. When monocytes and PMN were isolated independently from peripheral blood, only the monocytes exhibited constitutive annexin I binding. However PMN acquired the capacity to bind annexin I following co-culture with monocytes. PMN incubation with sodium azide, but not protease inhibitors, partially blocked this process. A similar increase in annexin I binding capacity was also detected in PMN following adhesion to endothelial monolayers. We propose that a juxtacrine activation rather than a cleavage-mediated transfer is involved in this process. Removal of annexin I binding sites from monocytes with elastase rendered monocytes functionally insensitive to full length annexin I or to the annexin I-derived pharmacophore, peptide Ac2–26, assessed as suppression of the respiratory burst. These data indicate that the annexin I binding site on phagocytic cells may have an important function in the feedback control of the inflammatory response and their loss through cleavage could potentiate such responses.

Key words: Lipocortin 1; Superoxide; Phagocyte; Glucocorticoid; Elastase; Adhesion

Introduction

Annexin I (also called lipocortin 1) is a member of a family of calcium and phospholipid-binding proteins termed 'annexins', particularly abundant in human and animal leukocytes. A combination of Western blotting, ELISA and flow cytometry analyses have found high levels of this protein in monocytes and polymorphonuclear leukocytes (PMN) whereas lower amounts were detected in lymphocytes.[1–3] Annexin I levels in circulating leukocytes are partially under the control of endogenous and exogenous glucocorticoid hormones. A single administration of anti-inflammatory steroids to human volunteers and experimental animals increases annexin I levels associated with circulating leukocytes.[1,4,5] Conversely removal of endogenous corticosterone by means of adrenalectomy or subchronic treatment with a steroid antagonist, mifepristone (RU486), reduces cell-associated annexin I levels by $\geq 50\%$.[5,6]

Recent observations have substantiated the role played by annexin I as a mediator of anti-inflammatory glucocorticoid hormones. Glucocorticoid-dependent annexin I externalization has been demonstrated in monocytes, macrophages and PMN.[4,7,8] Consistent with this, treatment of experimental animals with anti-annexin I antibodies prevented the anti-inflammatory effect of dexamathasone in several models of acute inflammation.[9,10] Besides glucocorticoid treatment, we and others have recently shown that annexin I secretion from human PMN occurs during the process of extravasation outside the blood vessel.[11,12] Much evidence now exists to support the hypothesis that annexin I interacts with the external face of the leukocyte plasma membrane to exert its anti-inflammatory properties, such as inhibition of superoxide anion generation from activated monocytes.[13]

Recent studies have brought to light the existence of specific annexin I binding molecule(s) on the cell surface of peripheral blood leukocytes and several other mono-myelocytic cell lines.[14] Incubation of a mixed population of peripheral blood leukocytes with different concentrations of annexin I resulted in a calcium-dependent and saturable binding to monocytes and PMN, but not to ad-mixed lymphocytes.[15] Monocyte binding capacity has been studied further, observing its sensitivity to trypsin treatment. In addition, monocytes lose annexin I binding capacity following incubation at 37°C in a time-dependent manner.[15] Further to our initial observations, specific annexin I binding to a subset of human T-lymphocytes

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and on rat anterior pituitary cells has also been described.[16,17]

Here we extend our previous observations. It was postulated that annexin I binding to leukocytes could be related to the anti-inflammatory profile of the protein, and that this could be modulated by the status of activation of the cells. To address this hypothesis we have investigated (i) the extent of annexin I binding to monocytes and PMN in relation to culture conditions, (ii) the susceptibility of annexin I binding to proteolytic enzymes, (iii) the connection between annexin I binding to monocytes and inhibition the respiratory burst.

Materials and methods

Leukocyte preparation and handling

Human peripheral blood was obtained from healthy volunteers by venepuncture. Healthy donors were selected on the basis of having no current illnesses nor receiving any form of medication. On average between 60 ml and 100 ml of blood was collected into 3.2% sodium citrate (10% v:v). Blood was diluted 1:1 with sterile phosphate-buffered saline (PBS) and separated on a discontinuous histopaque gradient (1077/1119) and centrifuged at 400 × *g* for 30 min at room temperature. Peripheral blood mononuclear cells (PBMC) and PMN layers were collected and cells washed three times in PBS. Erythrocytes were removed by hypotonic lysis in cold water. Finally, cells were resuspended in 1 ml RPMI 1640 medium supplemented with 0.2% BSA and 1.8 mM $CaCl_2$ (RBC). Cell aliquots were counted following 1:10 dilution in Turk's solution using a haematocytometer. PMN population purity was >97% with a distinct population being identified on the basis of the side scatter profile; in addition this population was CD14 negative. PMN contamination in the PBMC pellet was less than 3%. A discrete monocyte population was discerned by positivity for CD14.

Annexin I binding assay and flow cytometry analysis

Cells were resuspended at 50×10^6 cells/ml in RPMI 1640 containing 0.2% BSA (bovine serum albumin) and 1.8 mM $CaCl_2$ (RBC) and 20 µl aliquots were added to 96-well plates (Falcon) and incubated for 1 h at 4°C with 20 µl human recombinant full length annexin I at final concentrations ranging from 10 ng/ml to 60 µg/ml. The annexin I was prepared in PBC (PBS containing 0.2% BSA and 1.8 mM $CaCl_2$). Cells were then washed twice with PBC prior to addition of 20 µl human IgG (15 mg/ml in PBC) to block non-specific sites and 20 µl of a specific murine anti-human annexin I monoclonal antibody (mAb) 1B (60 µg/ml in PBC) and 1 h incubation at 4°C. Finally, cells were washed in PBC and incubated with 40 µl (1:30 dilution in PBC) of a $F(ab')_2$ fragment of a goat anti-mouse IgG conjugated to FITC. After a further 30 min incubation at 4°C the cells were washed and fixed in 1% paraformaldehyde. Samples were analysed within 48 h by flow cytometry (FACScan, Becton Dickinson, Oxford, UK). Cell populations were identified by their forward and 90° scatter characteristics, and fluorescence associated with each population (monocytes within the PBMC population, and PMN) was quantified in the FL1 channel. Cell-associated mean fluorescence intensity was converted into second antibody molecules bound per cell according to a calibration curve using standard fluorescence beads.[18]

As alluded to in previous publications,[15,19] this indirect methodology to assess annexin I binding to leukocytes was developed because radiolabelling protocols were found to be unsuitable for the protein, which degrades upon radiolabelling and does not retain either binding capacity or biological activity. An estimation of binding affinity was also made using a flow cytometric approximation of Scatchard analysis, in which free annexin I is calculated from total annexin I added to each tube less the amount bound to the cells.

In selected experiments monocyte populations were enriched (>80% pure) by Nycoprep™ gradient centrifugation (Nycomed Pharma AS, Oslo, Norway), and no difference was observed in the extent of annexin I binding to enriched monocytes when compared with monocytes ad-mixed with lymphocytes in the PBMC preparation (data not shown). For this reason thereafter monocytes were prepared as PBMC.

In some experiments additional cell surface markers on the monocyte plasma membrane were monitored with an anti-CD11b, anti-CD14 or an anti-CD54 mAb. All mAb were used at a 5 µg/ml final concentrations and incubated with the cells prior to staining with the $F(ab')_2$ fragment of a goat anti-mouse IgG conjugated to FITC (see above).

Selected samples were also analysed by confocal microscopy. FITC-stained cells were fixed with a equal volume of 2% paraformaldehyde in PBS and pelleted. Propidium iodide (PI) was added as a nuclear counterstain and cells were mounted onto glass slides and sealed with cover slips. A BioRad MRC600 confocal microscope system with 100× objective lens, 100 mW argon ion laser with an excitation line of 488 nm and COMOS™ analysis software was used to scan the samples and generate TIF format images.

Cell treatment and incubation conditions

Effect of PMN: monocyte co-incubation. PMN and monocyte populations isolated from peripheral blood were washed, counted and adjusted to 5×10^7 cells/

ml in RBC. Equal numbers of PMN and monocytes were co-incubated in polypropylene tubes. At the same time, PMN and monocytes were incubated separately. After a 15 min incubation period at room temperature, 20 µl of each treatment group (PMNs alone, monocytes alone and a mixed population of the two) were plated into each well (in duplicate). In some cases, either PMN or PBMC were treated with or without 0.2% sodium azide for 30 min at 37°C prior to 15 min co-incubation at room temperature.

Effect of leukocyte: endothelial cell co-incubation. Potential variation(s) in annexin I binding capacity of human PMN or monocytes was tested in an experimental system in which cell-cell interactions certainly occur. As recently described,[11] EA.hy926 cells[20] were grown to confluence in 6-well plates in DMEM-F12 supplemented with 10% FCS and antibiotics. After washing, 1.5×10^6 PMNs were added in 1 ml adhesion medium (Hanks's balanced salt solution containing 0.2% BSA, 1.3 mM $CaCl_2$ and $MgCl_2$; all reagents from Sigma Chemical Co., Poole, UK) and incubated with 0.1 µM platelet-activating factor (PAF) was used as a stimulus to increase the extent of cell adhesion.[21] After 30 min, non-adherent leukocytes were aspirated whereas adherent cells (PMNs + EA.hy926 or monocytes + EA.hy926 cells) were removed using 1 ml of non-enzymatic cell dissociation medium (Sigma).

Effect of protease inhibitors. In another set of experiments the annexin I binding capacity of a mixed population containing equal numbers of PMNs and monocytes were assessed following incubation with either cell type the specific elastase inhibitor ONO-5046 (10 µM) or a cocktail of protease and phosphatase inhibitors (phenylmethylsulphonyl fluoride (PMSF) 1 mM, leupeptin 0.2 mM, aprotinin 0.2 mM and trypsin inhibitor 1 mg/ml) for 30 min in a water bath at 37°C.

Effect of proteases. The effect of purified inflammatory proteolytic enzymes on the extent of annexin I binding was also evaluated. Type I collagenase (0.05, 0.5 and 5 U/ml), neutrophil-derived cathepsin G (1, 10 and 100 µg/ml), neutrophil-derived elastase (1, 10 and 100 µg/ml) or soybean trypsin (250 µg/ml) were added to the cells in polypropylene tubes at 37°C up to 30 min. In some experiments elastase was co-incubated with 10 µM ONO-5046, a selective catalytic inhibitor. At the end of the incubation period, proteolytic activity was removed by washing the cells twice in RPMI 1640 containing 4% BSA and 1.8 mM $CaCl_2$. Monocytes were subsequently washed once in protein-free RPMI 1640 and resuspended in RBC.

Effect of monocyte supernatants. Monocytes were incubated with or without 10 µg/ml human elastase for 30 min at 37°C. Reaction was stopped by a 5 min addition of 30 µM ONO-5046 followed by centrifugation at 2000 r.p.m. for 5 min. Supernatants were collected and co-incubated with control PMNs for 20 min at 37°C. Cells were spun once more prior to performing the annexin I binding assay.

Effect of monocyte or PMN activation. The effect of monocyte or PMN activation on the annexin I binding capacity displayed by each cell type was also investigated. For this purpose monocytes were incubated with 100 ng/ml monocyte chemotactic protein-1 (MCP-1) and PMNs were activated with 0.1 µM platelet-activating factor (PAF), 1 µM FMLP or 0.1 µM phorbol 12-myristate 13-acetate (PMA).

At the end of all these different treatments, cells were thoroughly washed in PBC, resuspended in RBC (20 µl per 10^6 cells) and the annexin I binding assay performed as described above.

Measurement of respiratory burst activity

Production of reactive oxygen species from activated monocytes was measured using the fluorochrome dihydrorhodamine 6G according to the manufacturer's indications. Monocytes isolated from peripheral blood were adjusted to 2.5×10^6 cells/ml in PBSGC (PBS containing 0.9% glucose and 1 mM $CaCl_2$). Aliquots (200 µl) were incubated with 5 mM dihydrorhodamine 6G for 10 min in a water bath at 37°C. After the incubation period monocytes were challenged with 0.1 µM PMA or 1 mg/ml opsonized zymosan for 10 min. The time and the concentrations were chosen on the basis of preliminary experiments. Opsonized zymosan was prepared by incubating 10 mg of boiled zymosan A with 1 ml of serum (prepared from the same donor) for 30 min in a water bath at 37°C, followed by two washes in PBSGC in a microfuge (3 min at 13 000 r.p.m). After the final incubation period the reaction was stopped by adding a final concentration of 0.2% sodium azide, this being followed by immediate incubation of cells on ice, and analysis by flow cytometry.

The biological efficacy of full length annexin I purified from human placenta and the annexin I-derived peptide Ac2–26[22,23] on this parameter of monocyte activation was then related to the binding capacity displayed by the cell. Human native annexin I (0.3–5 µg/ml) or peptide Ac2–26 (1–10 µg/ml) were added to the cells for 30 min prior to monocyte stimulation for a further 10 min with either PMA (0.1 µM) or opsonized zymosan (1 mg/ml). Also, the ability of annexin I and peptide Ac2–26 to suppress the respiratory burst was monitored in monocytes pretreated with 100 µg/ml elastase (30 min, 37°C), with the proteolytic activity being quenched with 10 µM ONO-5046 prior to washing and addition of peptide Ac2–26 (1 µg/ml) or annexin I (3 µg/ml).

Materials

Human annexin I and mAb IB,[24] used for the binding assays, was provided by Dr J.L. Browning (Biogen

Corporation, Cambridge, MA). Human biologically active full length native annexin I (used for the superoxide experiments) was purified from placenta and generously supplied by Dr E. Solito (INSERM, Paris, France). Human Ac2–26 (N-acetyl- AMVSEFLK-QAWFIENEEQEYVQTVK; Mr of 3050 Da, prepared at the Advance Biotechnology Centre, The Charing Cross and Westminster Medical School, London, UK) corresponds to a sequence comprising the majority of the human annexin I N-terminus region. The peptide was prepared by use of solid phase step-wise synthesis: amino acid composition and molecular weight were verified by mass spectrometry, and purity (=95%) was assessed by HPLC and capillary electrophoresis (data from manufacturer). Peptide solutions were always prepared in sterile PBS.

ONO-5046, a selective leukocyte elastase inhibitor,[25] was provided by ONO Pharmaceutical Co. (Osaka, Japan). Neutrophil-derived cathepsin G and elastase were from Calbiochem (Nottingham, UK). Dihydrorhodamine 6G was from Molecular Probes (Eugene, USA). Monoclonal antibodies anti-human CD11b (clone 44), anti-human CD14 (clone AML-2.23) and an anti-human CD54 (clone BC-14) were from Serotec Ltd (Oxford, UK). Beads bearing a known number of FITC molecules were purchased from Flow Cytometry Standards Co. (San Juan, PR, USA). Human MCP-1 protein was obtained from R&D Systems (Abingdon, UK).

Type I collagenase (from *Clostridium histolyticum*), soybean trypsin, zymosan A, PMA, FMLP and all other reagents were from Sigma Chemical Co. (Poole, Dorset, UK).

Statistics

Data are reported as mean ± SEM of n experiments performed in duplicate or triplicate. Approximate affinity constants (Kd) and maximal binding (Bmax) were determined using an flow cytometric approximation of Scatchard analysis. Briefly, in a seven point Scatchard plot, number of mols of free ligand were calculated as total mols added minus mols bound. Statistical differences between groups were analysed by analysis of variance followed by Bonferroni's test for post-hoc comparisons, or by two-way Student's t-test when only two groups were analysed using the Instat™ software. In all cases, a P value less than 0.05 was taken as the limit of significance.

Results

Monocytes and PMNs bind annexin I

When purified separately, human monocytes displayed a reproducible and significant annexin I binding capacity whereas enriched preparations (>95% pure) of PMN exhibited negligible binding

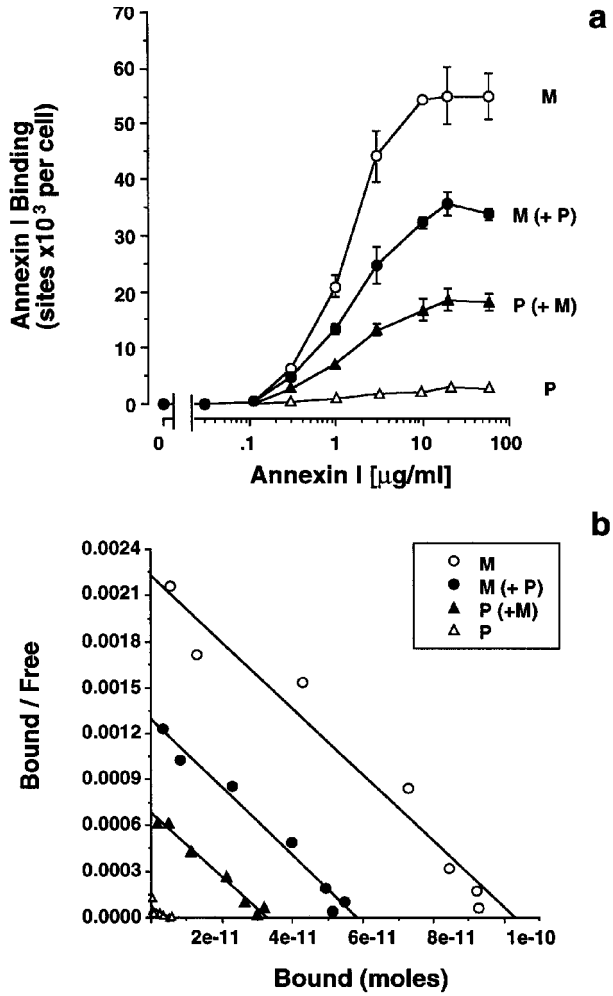
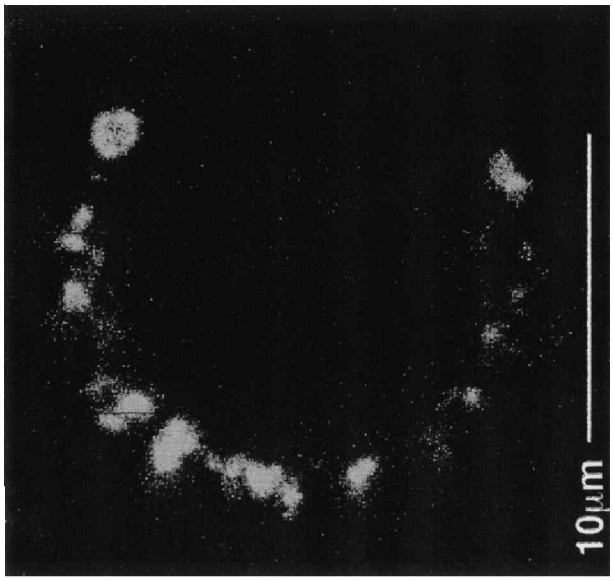


FIG. 1. Annexin I binding capacity and affinity of isolated and co-incubated monocytes (M) and polymorphonuclear leukocytes (P). Panel (a): Equal numbers of M and P were either co-incubated at room temperature for 15 min, or left separate, prior to washing and quantification of the annexin I binding capacity as described in the Experimental section. Data are mean ± SEM of one experiment performed in triplicate and are representative of four distinct experiments with similar results. Panel (b): Estimation of the dissociation constant (Kd) of annexin I binding by flow cytometry. Scatchard approximation as described in the Experimental section. Data are mean ± SEM as above.

(Fig. 1a). However, significant changes in binding capacities were found when the binding assay was performed with both cell types in co-culture (15 min at room temperature). Figure 1a illustrates a representative experiment where monocytes lost approximately 40% of annexin I binding capacity if co-cultured with PMN, and conversely the latter cell type acquired the capacity to bind annexin I. Cumulative data of n = 8 distinct experiments showed 42 ± 5% reduction of the extent of annexin I binding in monocytes which had been co-cultured with the PMN. In the same of set of experiments, PMN showed a substantial increase in annexin I binding such that 20 700 ± 2700 binding sites per cell were detected after co-incubation compared with 2300 ± 600



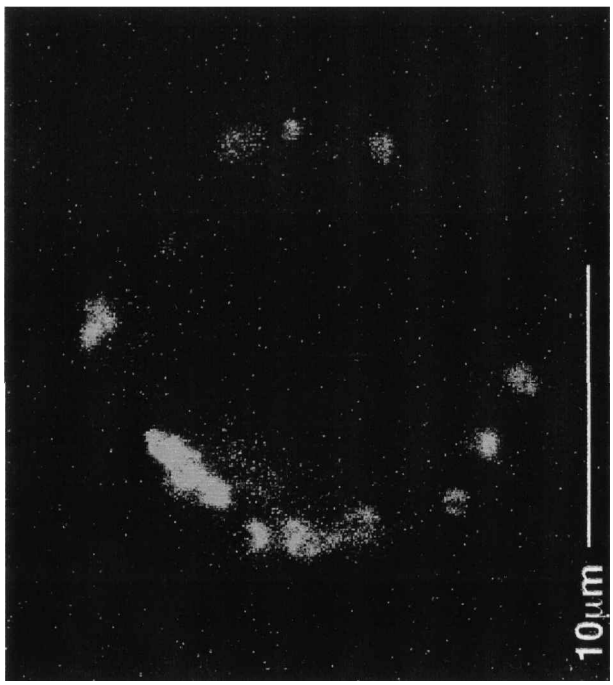


FIG. 2. Confocal images of peripheral blood monocytes demonstrating the punctate distribution of annexin I binding to the cell surface. The pattern of annexin I binding to two distinct monocytes is shown (green fluorescence). See Experimental section for details of staining and confocal analysis.

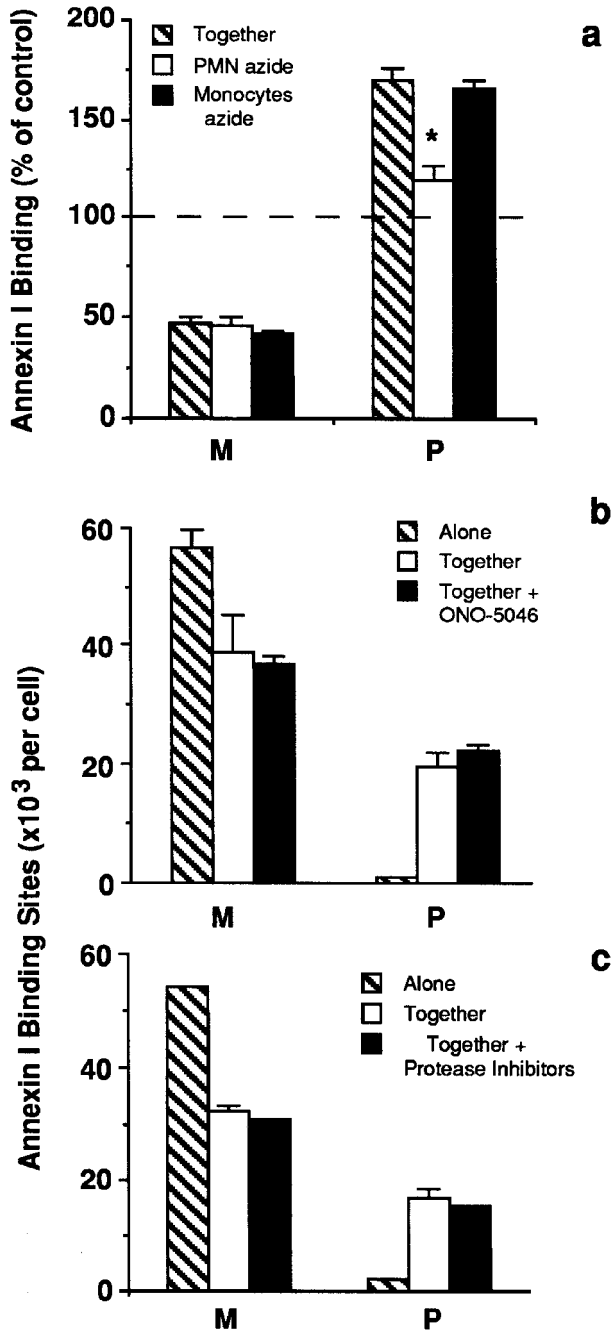


FIG. 3. Effect of several agents on annexin I binding capacity displayed by isolated and co-incubated monocytes (M) and polymorphonuclear leukocytes (P). Panel (a): M or P were treated separately with control buffer (no azide) or with 0.2% sodium azide for 30 min at 37°C prior to 15 min co-incubation at room temperature. Cells were then washed and binding capacity assessed using 20 μg/ml annexin I. Data are mean ± SEM of eight experiments performed in duplicate. $*P < 0.05$ as calculated on original values. Panel (b): Equal numbers of M and P were either kept separate, mixed together in the absence or presence of 10 μM ONO-5046 at room temperature for 30 min prior to washing and quantification of the annexin I binding capacity (with 20 μg/ml annexin I). Data are mean ± SEM of three experiments performed in duplicate. Panel (c) as in panel B with the difference that a cocktail of inhibitors (PMSF 1 mM, leupeptin 0.2 mM, aprotinin 0.2 mM and trypsin inhibitor 1 mg/ml) was used. Data are mean ± SEM of three experiments performed in duplicate.

binding sites quantified in absence of monocytes (using a saturating annexin I concentration of 20 μg/ml). Analyses of approximate affinity constant and total number of binding sites from four experiments performed with all annexin I concentrations are depicted in Fig. 1b. The following values were obtained: monocytes bound annexin I with an approximate Kd of 48 nM and a Bmax of 60 200 annexin I molecules per cell. Bmax but not Kd was reduced after incubation with PMN, at 37 700 binding sites per cell. The parameters for PMN were also

analysed after co-incubation with the monocytes with an approximate Kd of 47 nM and a Bmax of 19 700 annexin I molecules per cell.

Expression of another surface protein following monocyte and PMN co-incubation was also monitored. High levels of the CD14 antigen were found on isolated monocytes (293 200 ± 46 000 sites per cell) whereas much lower levels were detected on freshly isolated PMNs (4800 ± 1200 sites per cell). These values remained essentially unaltered following co-incubation, with 297 700 ± 32 200 and 5039 ± 2200 CD14 antigen sites per cell on monocytes and PMNs, respectively (data are mean ± SEM of n = 3 experiments performed in triplicate).

The appearance of annexin I binding was investigated by confocal microscopy. Figure 2 shows that exogenous annexin I bound to monocytes with a punctuate distribution on the cell surface. A similar pattern was also visualized on the PMN following incubation with monocytes (data not shown).

Next, we attempted to characterize the process by which monocytes and PMN respectively lost and gained annexin I binding capacity. Pre-incubation of PMN with sodium azide prior to co-incubation with the mononuclear cells prevented the acquisition of annexin I binding capacity by the PMN without altering the reduced binding capacity of the monocytes (Fig. 3a). Pre-incubation of monocytes with sodium azide had no effect. Addition of a specific elastase inhibitor (ONO-5046) or of a mixture of non-specific protease and phosphatase inhibitors failed to prevent either the reduction in monocyte binding or the PMN increase, in annexin I binding capacity (Fig. 3b and c).

Inflammatory proteases reduce annexin I binding to monocytes

Incubation of monocytes with trypsin abolished subsequent cell binding to exogenous annexin I, thus confirming previous observations.[15] Figure 4 shows that enzymes normally found in inflammatory exudates are also capable of reducing annexin I binding. Incubation of monocytes with elastase, cathepsin G or collagenase produced a significant reduction in the number of annexin I binding sites (Fig. 4a). The specificity of elastase effect was validated by using a selective inhibitor and addition of ONO-5046 prevented the loss of annexin I binding by elastase treatment (Fig. 4b).

The efficacy of elastase prompted the cell transfer experiment. Addition to PMNs of a supernatant derived from elastase-treated monocytes (in which the enzymatic activity had be quenched with ONO-5046) did not promote annexin I binding capacity: 3900 ± 950 and 3200 ± 640 annexin I binding sites per cell were measured after PMN incubation with an elastase-treated monocyte supernatant and control

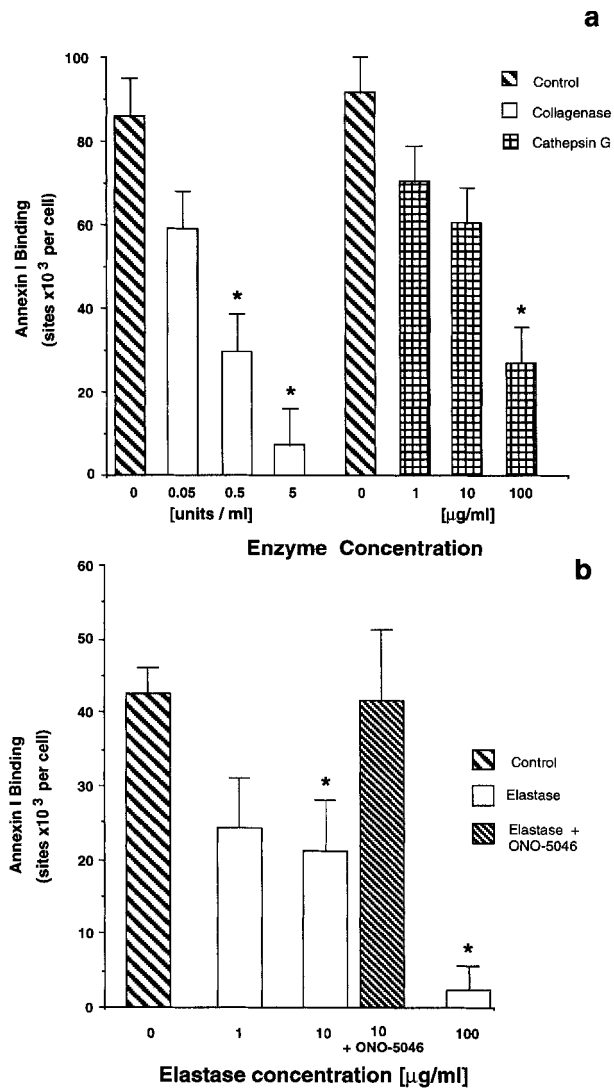


FIG. 4. Susceptibility of annexin I binding capacity displayed by human monocytes to inflammatory proteolytic enzymes. Monocytes were incubated with the reported concentrations of collagenase or cathepsin G (panel a), elastase ± 10 μM ONO-5046 (panel b) for 30 min prior to incubation with a saturable concentration of annexin I (10 μg/ml). Data are mean ± SEM of seven experiments (panel a) and six experiments (panel b) performed in duplicate. *P < 0.05.

monocyte supernatant, respectively (mean ± SEM of n = 2 experiments performed in triplicate).

The possibility that elastase, which binds to leukocyte plasma membrane,[26] could interfere with the binding by cleaving the ligand,[27] was also taken into account. Not only was ONO-5046 added to the incubation mixture prior to performing the annexin I binding assay (reported to block elastase activity in Fig. 4b), but also an annexin I preparation fully cleaved by elastase was tested. This preparation (33 Kd isoform) produced a curve of binding to monocytes comparable with that obtained using intact full length annexin I. For instance, 24 400 ± 170 and 24 500 ± 300 annexin I molecules bound per monocyte were found following incubation with

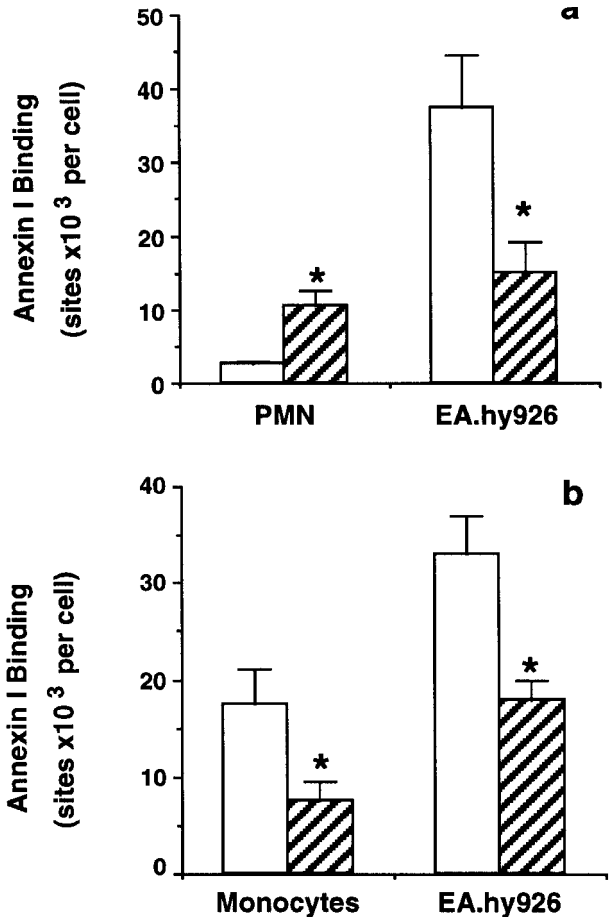


FIG. 5. Annexin I binding capacity in post-adherent PMNs and monocytes. Panel (a): Freshly prepared PMN or untreated EA.hy926 cells (open bars), or a mixture of PMN and EA.hy926 cells obtained after a 30 min adhesion period (hatched bars), were incubated with a saturating concentration of annexin I (10 μg/ml). *Panel b:* Freshly prepared monocytes or untreated EA.hy926 cells (open bars), or a mixture of monocytes and EA.hy926 cells (hatched bars) obtained after a 30 min adhesion period, were incubated with a saturating concentration of annexin I (10 μg/ml). In all cases, data are mean ± SEM from three experiment performed in triplicate. $*P < 0.05$ vs. untreated cells.

20 μg/ml intact full length annexin I or elastase-cleaved annexin I, respectively.

PMN, monocytes and endothelial cell interaction

Figure 5a shows that the modest annexin I binding capacity displayed by freshly prepared PMNs was greatly increased following adhesion to endothelial monolayers. This increase was not modified to any extent by cell-derived proteases which are likely to be present in this microenvironment, since a similar degree of annexin I binding was also attained when a mixture of non-specific protease inhibitors (see above) was added to the adhesion medium: this cocktail of protease inhibitors was internally validated since it prevented the reduced binding of annexin I to

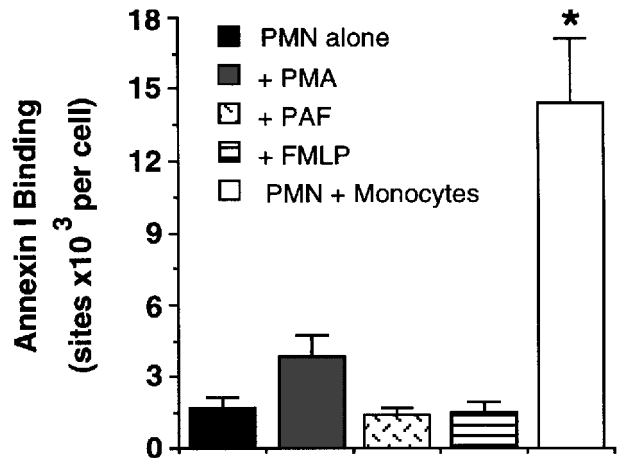


FIG. 6. Annexin I binding is not acquired by stimulated PMNs. PMNs were left untreated or were stimulated for 30 min with PMA (1 μM), PAF (0.1 μM) or FMLP (1 μM) prior to incubation with a saturating concentration of annexin I (10 μg/ml). As a positive control, the 'gain' of annexin I binding to PMN which had been co-incubated with monocyte (P + M group) was also monitored. Data are mean ± SEM of three experiments performed in triplicate. $*P < 0.05$.

post-adherent endothelial cells (data not shown). When monocytes were used in the same experimental procedure, a significant (~50%) reduction in annexin I binding capacity was displayed by post-adherent cells (Fig. 5b).

Effect of leukocyte activation

PMN activation either with 0.1 μM PAF, 1 μM FMLP or 1 μM PMA did not modify the annexin I binding capacity displayed by this cell type (Fig. 6). As expected, co-incubation of PMNs with monocytes increased annexin I binding to the neutrophils, and this was used as a positive control in this set of experiments. Similarly, monocyte activation with 100 ng/ml MCP-1 did not cause any reduction on the extent of annexin I binding to the cells (data not shown).

Biological significance of annexin I binding to human monocytes

Monocyte incubation with opsonized zymosan (1 mg/ml) or PMA (0.1 μM) produced a respiratory burst quantified by flow cytometry (Fig. 7a). This release was selectively inhibition by annexin I. Figure 7b shows a steep concentration-dependent curve for annexin I inhibition with a maximal effect ranging between 20% and 30% at 3 μg/ml. Annexin I inhibitory action was specific because the protein was unable to alter monocyte respiratory burst when cells were activated with PMA (Fig. 7b). The annexin I pharmacophore, peptide Ac2–26, was also able to inhibit superoxide production displaying selectivity towards opsonized zymosan (Fig. 7c).

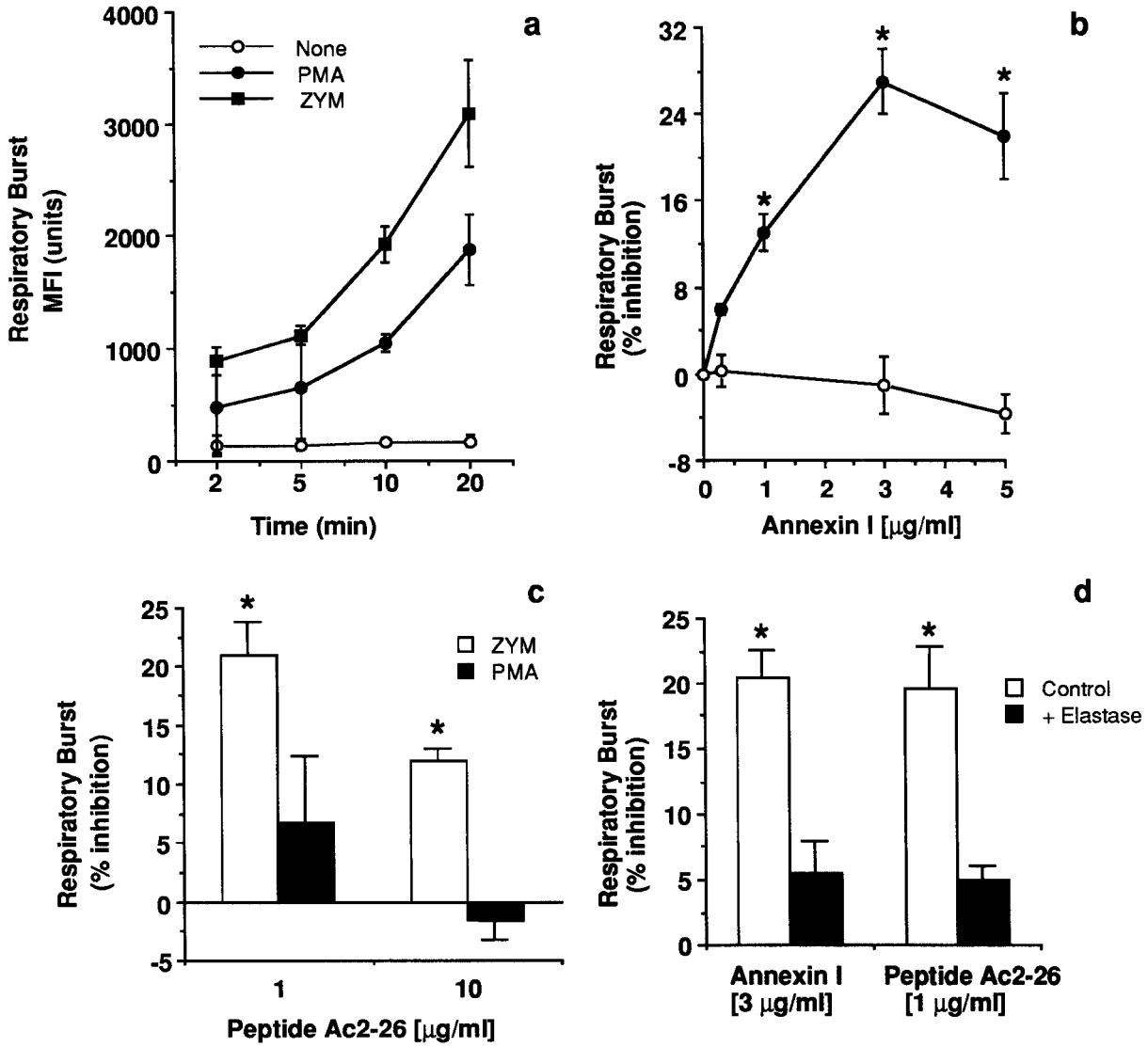


FIG. 7. Annexin I inhibition of respiratory burst of activated human monocytes is dependent on an intact binding site. Panel (a): Monocytes (5×10^5) were incubated with 0.1 μM PMA or 1 mg/ml opsonized zymosan (ZYM) for the reported times prior to flow cytometry. Data are mean ± SEM of eight experiments performed in duplicate. Monocytes (5×10^5) were incubated with the reported concentrations of annexin I (panel b) or peptide Ac2–26 (panel c) for 30 min prior to addition of 0.1 μM PMA (open circles) or 1 mg/ml opsonized zymosan (ZYM; closed circles) for a further 10 min. Data are mean ± SEM of five experiments performed in duplicate (*$P < 0.05$ as calculated on original values). Panel (d): Monocytes were treated with or without 100 μg/ml elastase for 30 min at 37°C. Reactions were stopped by adding 10 μM ONO-5046 and extensive washing in enzyme-free medium. Cells were then incubated with annexin I or peptide Ac2–26 as described above and stimulated with 1 mg/ml opsonized zymosan to activate the oxidative burst. Data are mean ± SEM of five experiments performed in duplicate (*$P < 0.05$ as calculated on original values).

Monocyte pre-treatment with elastase, a procedure known to reduce significantly annexin I binding (see Fig. 4), prevented the inhibitory action of annexin I and peptide Ac2-26 on zymosan-induced superoxide generation (Fig. 7d). The efficacy of elastase in reducing annexin I binding was also monitored. Binding was reduced by >95% following treatment of monocytes with 100 μg/ml elastase ($n = 4$ experiments, $P < 0.05$). The elastase effect was specific because the enzyme did not alter the extent of CD11b or CD54 expression on the monocyte plasma membrane (117 400 ± 8900 and 116 200 ± 6200 CD11b molecules per cell, and 6300 ± 300 and 6600 ± 300 CD54 molecules per cell in the absence or presence of elastase, respectively).

Discussion

The main conclusion from this study is that annexin I binding capacity expressed by resting monocytes is proteinaceous in nature and can be dramatically reduced following co-incubation with PMN. We also present strong evidence that occupation of annexin I binding sites on monocytes is a pre-requisite for the inhibitory biological actions exerted by this anti-inflammatory mediator.

As previously reported,[15,19] human monocytes display a saturable annexin I binding capacity following *in vitro* incubation with the human recombinant protein. Similarly, we confirmed that trypsin treatment of monocytes abolished their capacity to bind annexin I, and have shown for the first time that this binding is susceptible to the action of other more specific proteolytic enzymes. Both collagenase, cathepsin G and elastase treatment of monocytes resulted in almost complete loss of annexin I binding capacity. This is unlikely to be due to a destruction of the ligand,[27] since (i) cells were extensively washed in high BSA media; (ii) specific inhibitors were added to block the catalytic site of the protease, as in the case of elastase, and (iii) elastase-cleaved annexin I, generating a 33 Kd isoform, was able to bind to monocytes to a similar degree as intact annexin I.

However, this binding is vulnerable in the presence of PMN. In previous studies PMN were always co-incubated with monocytes in the binding assay, and showed a binding capacity with a Kd similar to the one calculated for monocytes.[19] Using purified cultures, two distinct processes can be distinguished: monocytes (either pure or together with lymphocytes), but not PMN, express constitutive annexin I binding capacity, which however can be differentially modified in co-culture conditions. PMN acquired annexin I binding capacity *only* after co-incubation with the monocytes, which in turn showed a comparable degree of loss. A similar Kd could be calculated for both cell types. On this basis, we suggest that annexin I binds to an identical 'site' on both leukocyte types.

There are two potential ways by which the annexin I binding sites on the two cell types could be altered: either the binding site is released (by a proteolytic event) in the culture medium and adheres to the PMN, or it is differentially expressed as a consequence of activation phenomena occurring between the adjacent monocyte and PMN. Examples of the latter, i.e. cell activation through a juxtacrine mechanism, have been recently reported.[28]

Based on the experimental data obtained, we believe that the first hypothesis is unlikely for at least three reasons. Firstly, despite the susceptibility showed by the monocyte annexin I binding capacity to proteolytic enzymes, the loss observed after co-incubation with the PMNs was not blocked by a mixture of non-specific protease inhibitors or by the specific elastase inhibitor ONO-5046. Secondly, addition to PMNs of a supernatant from elastase-treated monocytes was without effect. Thirdly, monocyte activation with the specific chemoattractant and activator MCP-1 did not reduce the annexin I binding capacity displayed by these cells. Similarly, PMNs did not increase their capacity to bind annexin I following incubation with PAF, FMLP or PMA. All these data rule out the possibility that proteolytic activities, transiently exposed on the PMN plasma membrane,[26] could be responsible for this phenomenon.

However, an active role for the PMN was suggested by the experiments with sodium azide. Blockade of PMN, but not monocyte, respiratory pathways prevented the phenomenon of annexin I binding acquisition by the neutrophil without affecting the loss from the monocyte. The hypothesis of a juxtacrine process is clearly reinforced by the experiments of PMN interaction with endothelial cells. Adherent PMNs acquired a considerable binding capacity compared to control cells. This was clearly due to the adhesion process, and not to the addition of FMLP, since this cell activator *per se* is totally inactive of PMN in suspension. In this context, it is interesting to note that following PMN adhesion to endothelial monolayers, endogenous annexin I is externalized on the surface of the adherent leukocytes.[11] It therefore seems that the entire annexin I pathway can be fully activated at the interface between adherent PMN/endothelial cells.

In conclusion we propose that PMN co-incubation with monocytes results in acquisition of annexin I binding as a result of an active phenomenon due to a juxtacrine mechanism and involving new protein expression on the plasma membrane. It remains unclear how the annexin I binding protein could disappear from the monocyte plasma membrane. It seems unlikely that this is the result of a proteolytic process, but further studies are necessary to determine if this is the result of internalization following juxtacrine activation in the presence of PMN.

Annexin I possesses the biological profile of an inhibitory protein with the ability to suppress selected parameters of cell activation, including superoxide anion generation and arachidonate, eicosanoids and nitric oxide release.[13,29,30] The annexin I-derived N-terminus peptide, peptide Ac2–26, has been reported to inhibit arachidonate and elastase release from activated PMNs[21] and macrophage phagocytosis.[31] In the present study we report for the first time that annexin I, and its pharmacophore peptide Ac2–26, must interact with proteins associated with the monocyte plasma membrane in order to suppress cell activation, measured as respiratory burst. The inhibition was stimulus-dependent, such that when cells were activated with PMA, which bypasses receptor-mediated phenomena by activating intracellular protein kinases, no effect was exhibited by annexin I or peptide Ac2–26. A maximal inhibition of 20–30% was reached in this set of experiments which is similar to that obtain with peptide Ac2–26 on murine macrophage phagocytosis using the same protocol.[31] Importantly, annexin I showed an approximate IC50 of 1.2 μg/ml (32 nM) which is close to the Kd calculated from the binding experiments. Pretreatment of monocytes with elastase did not affect the extent of cellular respiratory burst following opso-

nized zymosan or the degree of expression of specific surface markers (CD11b and CD54). In contrast, elastase abrogated annexin I binding to monocytes and similarly abolished the inhibitory action displayed by annexin I or its N-terminus peptide. This strongly suggests that annexin I binding to the protein(s) on the monocyte plasma membrane[15] is instrumental in its inhibitory properties. In addition, peptide Ac2–26 action in an *in vivo* model of experimental inflammation was already proposed to require an interaction with the annexin I binding site,[22] and this hypothesis in reinforced by the data here presented on activated monocytes *in vitro*.

In conclusion, we report an exquisite sensitivity of the annexin I binding protein(s) present on human monocytes to the phenomena of activation and provide strong evidence that the binding is a prerequisite to the anti-inflammatory action produced by annexin I on this cell type.

References

1. Goulding NJ, Godolphin JL, Sharland PR, Peers SH, Sampson M, Maddison PJ, Flower RJ. Anti-inflammatory lipocortin 1 production by peripheral blood leucocytes in response to hydrocortisone. *Lancet* 1990; **335**: 1416–1418.
2. Morand EF, Hutchinson P, Hargreaves A, Goulding NJ, Boyce NW, Holdsworth S. Detection of intracellular lipocortin 1 in human leukocyte subsets. *Clin Immunol Immunopathol* 1995; **76**: 195–202.
3. Ernst JD. Annexin functions in phagocytic leukocytes. In: Seaton BA, ed. *Annexins: Molecular Structure to Cellular Function*. Austin, TX: R. G. Landes, 1996: 81–96.
4. Mancuso F, Flower RJ, Perretti M. Leukocyte transmigration, but not rolling or adhesion, is selectively inhibited by dexamethasone in the hamster post-capillary venule. Involvement of endogenous lipocortin 1. *J Immunol* 1995; **155**: 377–386.
5. Perretti M, Flower RJ. Measurement of lipocortin 1 levels in murine peripheral blood leukocytes by flow cytometry: modulation by glucocorticoids and inflammation. *Br J Pharmacol* 1996; **118**: 605–610.
6. Vishwanath BS, Frey FJ, Bradbury M, Dallman MF, Frey BM. Adrenalectomy decreases lipocortin-I messenger ribonucleic acid and tissue protein content in rats. *Endocrinology* 1992; **130**: 585–591.
7. Peers SH, Smillie F, Elderfield AJ, Flower RJ. Glucocorticoid- and non-glucocorticoid induction of lipocortins (annexins) 1 and 2 in rat peritoneal leucocytes *in vivo*. *Br J Pharmacol* 1993; **108**: 66–72.
8. Coméra C, Russo-Marie F. Glucocorticoid-induced annexin I secretion by monocytes and peritoneal leukocytes. *Br J Pharmacol* 1995; **115**: 1043–1047.
9. Perretti M, Flower RJ. Modulation of IL-1-induced neutrophil migration by dexamethasone and lipocortin 1. *J Immunol* 1993; **150**: 992–999.
10. Flower RJ, Rothwell NJ. Lipocortin-1: cellular mechanisms and clinical relevance. *Trends Pharmacol Sci* 1994; **15**: 71–76.
11. Perretti M, Croxtall JD, Wheller SK, Goulding NJ, Hannon R, Flower RJ. Mobilizing lipocortin 1 in adherent human leukocytes downregulates their transmigration. *Nature Med* 1996; **22**: 1259–1262.
12. Vergnolle N, Coméra C, Buéno L. Annexin 1 is overexpressed and specifically secreted during experimentally induced colitis in rats. *Eur J Biochem* 1995; **232**: 603–610.
13. Maridonneau-Parini I, Errasfa M, Russo-Marie F. Inhibition of O_2^- generation by dexamethasone is mimicked by lipocortin I in alveolar macrophages. *J Clin Invest* 1989; **83**: 1936–1940.
14. Ahluwalia A, Buckingham JC, Croxtall JD, Flower RJ, Goulding NJ and Perretti M. The biology of annexin I. In: Seaton BA, ed. *Annexins: Molecular Structure to Cellular Function*. Austin, TX: R.G. Landes, 1996: 161–199.
15. Goulding NJ, Pan L, Wardwell K, Guyre VC, Guyre PM. Evidence for specific annexin I-binding proteins on human monocytes. *Biochem J* 1996; **316**: 593–597.
16. Kim HW, Choi E, Yoo B, Choi JR, Park YM, Lee SO, Moon H-M, Na DS. Lipocortin 1 binding sites on human T-cells: the population of cells with the binding sites is larger in CD8+ T-lymphocytes than CD4+ T-lymphocytes. *Biochem Mol Biol Int* 1996; **40**: 1167–1173.
17. Christian HC, Taylor AD, Flower RJ, Morris JF, Buckingham JC. Characterization and localization of lipocortin 1-binding sites on rat anterior pituitary cells by fluorescence-activated cell analysis/sorting and electronic microscopy. *Endocrinology* 1998; **138**: 5341–5351.
18. Le Bouteiller PP, Mishal Z, Lemonnier F, Kourilsky FM. Quantification by flow cytofluorometry of HLA class I molecules at the surface of murine cells transformed by cloned HLA genes. *J Immunol Methods* 1983; **61**: 301–315.
19. Goulding NJ, Luying P, Guyre PM. Characteristics of lipocortin 1 binding to the surface of human peripheral blood leucocytes. *Biochem Soc Trans* 1990; **18**: 1237–1238.
20. Edgell C-J, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci USA* 1983; **80**: 3734–3737.
21. Perretti M, Wheller SK, Choudhury Q, Croxtall JD, Flower RJ. Selective inhibition of neutrophil function by a peptide derived from lipocortin 1 N-terminus. *Biochem Pharmacol* 1995; **50**: 1037–1042.
22. Perretti M, Ahluwalia A, Harris JG, Goulding NJ, Flower RJ. Lipocortin-1 fragments inhibit neutrophil accumulation and neutrophil-dependent edema in the mouse: a qualitative comparison with an anti-CD11b monoclonal antibody. *J Immunol* 1993; **151**: 4306–4314.
23. Perretti M. Lipocortin-derived peptides. *Biochem Pharmacol* 1994; **47**: 931–938.
24. Pepinsky RB, Sinclair LK, Dougas I, Liang C-M, Lawton P, Browning JL. Monoclonal antibodies to lipocortin-1 as probes for biological functions. *FEBS Lett* 1990; **261**: 247–252.
25. Iwamura H, Moore A, Willoughby DA. Interaction between neutrophil-derived elastase and reactive oxygen species in cartilage degradation. *Biochim Biophys Acta* 1993; **1156**: 295–301.
26. Owen CA, Campbell MA, Sannes PL, Boukedes SS, Campbell EJ. Cell surface-elastase and cathepsin G on human neutrophils: a novel, non-oxidative mechanism by which neutrophils focus and preserve catalytic activity of serine proteinases. *J Cell Biol* 1995; **131**: 775–789.
27. Huang K-S, McGray P, Mattaliano RJ, Burne C, Chow EP, Sinclair LK, Pepinsky RB. Purification and characterization of proteolytic fragments of lipocortin I that inhibit phospholipase A_2. *J Biol Chem* 1987; **262**: 7639–7645.
28. Zimmerman GA, Lorant DE, McIntyre TM, Prescott SM. Juxtacrine intercellular signaling: another way to do it. *Am J Respir Cell Mol Biol* 1993; **9**: 573–577.
29. Cirino G, Flower RJ, Browning JL, Sinclair LK, Pepinsky RB. Recombinant human lipocortin 1 inhibits thromboxane release from guinea-pig isolated perfused lung. *Nature* 1987; **328**: 270–272.
30. Wu C-C, Croxtall JD, Perretti M, Bryant CE, Thiemermann C, Flower RJ, Vane JR. Lipocortin 1 mediates the inhibition by dexamethasone of the induction by endotoxin of nitric oxide synthase in the rat. *Proc Natl Acad Sci USA* 1995; **92**: 3473–3477.
31. Getting SJ, Flower RJ, Perretti M. Inhibition of neutrophil and monocyte recruitment by endogenous and exogenous lipocortin 1. *Br J Pharmacol* 1997; **120**: 1075–1082.

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