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Mechanisms of pertussis toxin-induced myelomonocytic cell adhesion: role of Mac-1(CD11b/CD18) and urokinase receptor (CD87)

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SUMMARY

Stimulation of monoblastic U937 cells with transforming growth factor β1 and 1,25-$(OH)_2$ vitamin D_3 (TGF-β1/D_3) upregulates urokinase receptor (uPAR) and confers urokinase-dependent adhesiveness to the cells for serum- or vitronectin-coated surfaces. Recent studies show that uPAR itself is a high-affinity adhesion receptor for vitronectin and that urokinase (uPA) is an activator of this adhesive function. In the course of exploring possible G-protein involvement in this adhesion it was observed that TGF-β1/D_3-primed U937 cells became adhesive to vitronectin in an uPAR-dependent manner when exposed to pertussis toxin (PTX). The adherent response is concentration- and time-dependent, and was not due to the ADP-ribosyltransferase activity of the toxin because the purified B-subunit of PTX was equally effective. Although promoting adhesion to serum- or vitronectin-coated surfaces, PTX blocked spontaneous cell adhesion to fibrinogen, an endogenous ligand for the Mac-1 receptor (CD11b/CD18). Flow cytometry study showed that expression of the α-subunit of Mac-1 (CD11b) on primed cells was increased by nearly threefold. Monoclonal antibody to CD11b abolished the PTX-induced cell adhesion and the binding of the primed cells to PTX-coated plates. Activation of Mac-1 receptor by its endogenous ligand fibrinogen induced cell adherent response similar to PTX. PTX, but not uPA, triggered a rapid rise in $[Ca^{2+}]_i$ in primed U937 cells, and PTX-induced adhesion was significantly attenuated by 1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid/acetoxy-methyl ester (BAPTA/AM), a selective membrane-permeant $[Ca^{2+}]_i$ chelator. PTX-induced cell adhesion was also prevented by antibodies to uPAR and by conditioned medium containing soluble uPAR. Together these data indicate that PTX B-subunit may bind to Mac-1 integrin, which leads to a rapid rise in $[Ca^{2+}]_i$ and subsequent activation of uPAR for adherence to vitronectin, suggesting a functional link between Mac-1 and activation of uPAR important to cellular trafficking and host defence in response to *Bordetella pertussis* infection.

INTRODUCTION

Pertussis toxin (PTX) is the major virulence factor for the pathogenesis of respiratory tract infection by *Bordetella pertussis*. PTX is a heterohexamer which is functionally divided into A and B subunits analogous to cholera toxin. The A-subunit consists of a single polypeptide which possesses ADP-ribosyltransferase activity, and the B-subunit confers binding specificity to the target cell membrane. Upon prolonged incubation of certain cells with PTX, such as neutrophils, the A-subunit is internalized and ADP-ribosylates the α subunit of the membrane bound Gi-like protein, thus leading to blockade of certain transmembrane signalling processes, and eventually cellular intoxication.[1–3]

In addition to its delayed inhibitory effect on the Gi protein, PTX has also been shown to elicit rapid cellular responses in several human cell lines. In human T lymphocytes, PTX triggers an immediate rise in $[Ca^{2+}]_i$, an elevation of cellular

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Abbreviations: uPA, urokinase; uPAR, urokinase receptor; PTX, pertussis toxin; DFP-uPA, diisopropylfluorophosphate-inactivated uPA; RGD, Arg-Gly-Asp; TGF-β1, transforming growth factor type-β1; D_3, 1,25$(OH)_2$-vitamin D_3; $[Ca^{2+}]_i$, intracellular calcium concentration; mAb, monoclonal antibody; CTX, cholera toxin; BAPTA/AM, 1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid/acetoxy-methyl ester; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; FBS, fetal bovine serum.

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diacylglycerol and inositol trisphosphate (IP_3) levels, and an increase in tyrosine phosphorylation of certain cellular proteins.[4–7] Moreover, PTX rapidly promotes human platelet aggregation and accumulation of IP_3 and phosphatidic acid.[8] All these cellular responses can be reproduced by the purified B-subunit moiety alone, suggesting that the non-catalytic binding domain of PTX is important in mediating these early cellular events. However, the binding site(s) for the PTX B-subunit to elicit these rapid cellular responses have not been unequivocally identified.

Stimulation of myelomonocytic cells with vitamin D_3 and/or cytokines *in vitro* results in expression of a macrophage profile of cell surface receptors including the integrins lymphocyte function associated antigen (LFA)-1 (CD11a/CD18), Mac-1 (CD11b/CD18), p150, 95 (CD11c/CD18), as well as the complement receptor CR1, Fc receptors, and uPAR, among others.[9–13] Our recent work has shown that urokinase (uPA) can induce transforming growth factor β1 and vitamin D_3 (TGF-β1/D_3)-primed cells to adhere to serum- or vitronectin-coated plates.[10,14] More recently, we have identified the uPAR (CD87) itself as a high affinity adhesion receptor for the matrix form of vitronectin.[15] The acquisition of adhesive properties in blood-borne myelomonocytic cells is an essential step to enable cells to migrate through endothelium and extracellular matrices as part of the inflammatory response. The mechanism of uPA-mediated activation of the uPAR for vitronectin binding is unknown. In the course of investigating this phenomenon, we used PTX as a probe for potential G protein involvement. As a result, we discovered that PTX, rather than an inhibitor, was an active inducer of myeloid cell adhesion to vitronectin.

In the present study, we found that PTX B-subunit triggers a rapid rise in $[Ca^{2+}]_i$ in TGF-β1/D_3-primed U937 cells followed by an adherent response. This adherent response occurs via binding of PTX B-subunit to Mac-1 integrin and subsequent activation of uPAR for vitronectin adhesion, suggesting a functional link between Mac-1 and uPAR in myelomonocytic cells in response to *B. pertussis* infection.

MATERIALS AND METHODS

Reagents

RPMI-1640 was purchased from Mediatech (Washington, DC). Fetal bovine serum was from Hyclone Laboratories (Logan, UT). Mouse monoclonal anti-$\alpha_V\beta_5$ antibody (used as ascites fluid), penicillin G, streptomycin sulphate, and HEPES were obtained from Life Technologies, Inc. (Grand Island, NY). Tissue culture ware was from Falcon Labware (Lincoln Park, NJ). Transforming growth factor-β1 (TGF-β1) was from Collaborative Research Inc. (Bedford, MA) and 1,25-$(OH)_2$ vitamin D_3 was a gift of Dr M. Uskokvic of Hoffman-LaRoche Laboratories (Nutley, NJ). Purified PTX was a gift of Dr George Siber of Massachusetts Public Health Biologic Laboratories (Boston, MA) and was prepared as described.[4] B-subunit of PTX was purchased from List Biological Laboratories (Campbell, CA). Recombinant human two-chain high molecular weight uPA was a gift of Drs Jack Henkin and Andrew Mazar of Abbott Laboratories (Abbott Park, IL) and uPA was inactivated by diisopropylfluorophosphate (DFP) as described.[10] Mouse ascites IgG_1 (MOPC-21), ionomycin, A23187, and probenecid were obtained from Sigma Chemical Co. (St Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated, affinity-purified goat anti-mouse immunoglobulin G (IgG) (H + L), F(ab')$_2$ antibody and Arg-Gly-Asp (RGD) fragment were from Boehringer Mannheim Corporation (Indianapolis, IN). Mac-1 blocking mouse antibody directed to CD11b (Clone 2LPM19c, used as culture supernatant) was from Dako Corporation (Carpinteria, CA). VIM5 (used as ascites fluid, previously being used under the name MA13) was a monoclonal antibody (mAb) provided by Dr W. Knapp of University of Vienna, as part of a panel of mAbs to uPAR for the 5th International Workshop on Leukocyte Differentiation Antigen, 1993. VIM5 was determined to be a potent inhibitor of myeloid cell adhesion and that this inhibition of adhesion could be overcome by adding excess uPA to compete with the antibody for receptor binding.[16] [^{3}H]thymidine was purchased from Du-Pont New England Nuclear (Boston, MA). Human fibronectin and mouse anti-CD11c (p150, 95; used as purified IgG) antibody were from Becton Dickinson (Bedford, MA). Human fibrinogen (free of plasminogen, vWf, and fibronectin) was from Enzyme Research Laboratories, Inc. (South Bend, IN). Vitronectin was prepared according to the method of Yatohgo *et al.*[17] BAPTA/AM and Fluo-3/AM were obtained from Molecular Probes (Junction City, OR).

Cells

All experiments in this study were performed using the monocytic leukemic U937 cell line from American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 supplemented to 10% (v/v) with fetal bovine serum (FBS), 20 mM HEPES (pH 7·4), 100 U/ml penicillin G, and 100 μg/ml streptomycin in a humidified incubator with 5% CO_2 at 37°. Before any experimentation, cells were seeded at 10^6/ml and were differentiated to monocyte/macrophage with 1 ng/ml TGF-β1 and 50 nM 1,2-$(OH)_2$ vitamin D_3 (TGF-β1/D_3) for 24 hr.[4] Cells without TGF-β1/D_3 pretreatment were used as controls.

Adhesion assays

Adherent cells were assayed by either a radiometric or colorimetric method. For the radiometric assay, cells at 10^6/ml were labelled by incubation with 1 μCi/ml [^{3}H]thymidine for 24 hr followed by a wash with serum-free RPMI to remove excess [^{3}H]thymidine. After centrifugation, the cells were resuspended at the original density in RPMI containing 10% FBS with or without TGF-β1/D_3 pretreatment for another 24 hr. Cells were then resuspended in fresh RPMI medium containing 10% FBS and treated with PTX or diisopropylfluorophosphate (DFP)-inactivated uPA (DFP-uPA). Aliquots of 100 μl of cells (1×10^5) per well were placed in duplicate in 96-well microtitre plates for 1 hr at 37°. Blockers or mAbs were added to the cell suspension at various times prior to the treatment with PTX or DFP-uPA. The contents of control wells were collected for determination of total c.p.m. per well before plates were immersed in normal saline. Non-adherent cells were flicked out and adherent cells were lysed in 50 μl lysis buffer (10% glycerol, 0·2% sodium dodecyl sulphate, 0·2% Triton X-100) and counted in a liquid scintillation counter. Results were expressed as the percentage of total c.p.m. of the control wells. A colorimetric assay was also employed and the procedures were similar to radiometric method except that the adherent cells were fixed in methanol for 15 min and then

stained with Geimsa stain for 1 hr. Absorbance at 550 nm was measured using a Bio-Rad EIA reader Model 2550. Data were expressed as percent of maximum adherent responses of respective sets of treatment.

To examine the role of $[Ca^{2+}]_i$ on PTX-induced cell adhesion, cells were loaded with 1,2-bis-(2-aminophenoxy)-ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid/acetoxy-methyl ester (BAPTA/AM) at a final concentration of 25 μM in RPMI for 1 hr followed by two washes to remove excess BAPTA/AM. Cells were then resuspended in RPMI containing 10% FBS and 20 μM probenecid was added to prevent efflux of the organic anion BAPTA.[4] Cells were treated with PTX and adhesion assay was performed as above. To determine the selective effects of PTX on myeloid cell adhesion to various matrix glycoproteins, aliquots of 100 μl of vitronectin (5 μg/ml), fibronectin (20 μg/ml), or fibrinogen (100 μg/ml) in phosphate-buffered saline (PBS) were added to 96-well plates and were allowed to coat the wells overnight at 4°. After blocking the coated wells with 1% BSA for 2 hr at 37°, experiments were then conducted as described above in the absence of FBS. To determine the binding of U937 cells to PTX, 96-well microtitre plates were coated with aliquots of 100 μl of PTX (5 μg/ml) in PBS and the experiment was conducted as described for PTX-induced selective adherent responses to various matrix proteins. To determine the effect of soluble uPAR (see below for the preparation of soluble uPAR) on PTX-induced myeloid cell adhesion, cells were suspended in conditioned medium containing 10% FBS with or without the soluble uPAR and adhesion assays were performed as described. To determine if activation of Mac-1 in these primed U937 cells would lead to adherent response, adhesion assays were performed using fibrinogen (1 mM), an endogenous ligand for Mac-1 receptor.

Preparation of soluble uPAR

Human kidney epithelial 293 cells were transfected with a truncated, soluble version (residue 1-277) of human uPAR as described by Wei *et al.*[15] Transfected cells were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 10% FBS and 50 μg/ml hygromycin. Soluble uPAR was concentrated (~12 nM) from conditioned medium collected after an overnight culture of the transfected cells in DMEM devoid of serum and hygromycin and was shown to bind to uPA. Conditioned medium obtained from non-transfected 293 cells or from cells transfected with an irrelevant gene was used as control.

Intracellular Ca^{2+} measurements

Primed U937 cells were loaded with the $[Ca^{2+}]_i$ indicator dye Fluo-3/AM (10 μM) for an hour at 37° in RPMI and washed three times with a buffer consisting of 140 mM NaCl, 4 mM KCl, 1·0 mM KH_2PO_4, 1·8 mM $CaCl_2$, 0·8 mM $MgCl_2$, 10 mM glucose, and 25 mM HEPES (pH 7·4).[4] Cells were then resuspended with this buffer at 10^7 cells/ml. To prevent efflux of the organic anion Fluo-3 from the cells, probenecid at a final concentration of 10 μM was added to the cells. An aliquot of 50 μl cell suspension was transferred to cuvette containing 2 ml buffer with continuous stirring and temperature constantly maintained at 37°. For experiments conducted in the absence of extracellular Ca^{2+}, EGTA (3 mM) was added to the cells 30 sec before the addition of mitogen. To determine if PTX-induced $[Ca^{2+}]_i$ rise is mediated by binding to Mac-1, 2LPM19c (diluted 1 : 30) was added to the cells 10 min before addition of PTX (5 μg/ml). Ca^{2+}-specific fluorescence was measured by a Perkin-Elmer LS5 spectrofluorometer with excitation wavelength at 506 nm and emission wavelength at 526 nm. The excitation and emission slit widths were set at 5 and 10 nm, respectively. Maximum fluorescence value was achieved by addition of NP-40 and ionomycin, and minimum fluorescence value was attained by addition of EGTA and $MnCl_2$. A K_d of 320 nM of Fluo-3 for Ca^{2+} was used to calculate $[Ca^{2+}]_i$ as described.[18]

Flow cytometry

Cells (5×10^5) were suspended in 200 μl of RPMI containing 2·5% FBS and were treated with PTX (5 μg/ml) for 15 min at 37°. After washing, cells were resuspended in 200 μl of the same medium, and Fc receptors were blocked by addition of human serum at a final concentration of 1%. The primary monoclonal antibody (mAb) was then added to cell suspension followed by incubation on ice for 30 min. The cells were washed twice in RPMI/2·5% FBS and then resuspended in 200 μl of the same medium with FITC-conjugated goat anti-mouse $F(ab')_2$ fragments and incubated for another 30 min on ice. Cells were washed twice in cold fluorescence-activated cell sorter (FACS) buffer and then were brought to a concentration of 10^6/ml. The mean fluorescence value of the live cell population was determined by measuring mean fluorescence of 3000 cells on an Ortho Flow Cytometry System 2150 equipped with computer interface from Cytomation and air-cooled argon laser beam. Excitation wavelength was set at 488 nm and emission was at 550 nm. Non-specific fluorescence was determined on cells incubated with a mouse ascites IgG_1 isotype having irrelevant antigen specificity.

RESULTS

PTX-induced myeloid cell adhesion

Human monoblastic U937 cells have been shown to differentiate to a monocyte/macrophage phenotype after TGF-β1/D_3 treatment for 24 hr. These cells become adhesive in response to uPA in a concentration- and time-dependent manner.[10,14] In the course of investigating the possible involvement of heterotrimeric G proteins in this process, we added PTX to see if it inhibited uPA-stimulated adhesion. Surprisingly, these same differentiated U937 cells also became adherent in response to PTX in the absence of exogenously added uPA. Among many stimulants tested in this cell adhesion model, including epidermal growth factor, thrombin,[10] *N*-formyl-met-leu-phe, C5a, platelet activating factor, and cholera toxin, only PTX was able to produce an adherent response of primed U937 cells analogous to uPA. This observation was characterized by a series of experiments detailed below.

Although cytokine-treated cells demonstrated macrophage morphology, they were not adherent (< 10%) in serum unless PTX was added (Fig. 1a). The threshold concentration for PTX to induce myeloid cell adhesion was at 0·5–1 μg/ml (5–10 nM) and maximum adhesion was reached at 5 μg/ml (48 nM). The EC_{50} value was 2 μg/ml (20 nM). A time–course study showed that, within 15 min after PTX treatment, up to 80% of maximal cell adhesion had been achieved and maximum adhesion was attained by 30–60 min (Fig. 1b). Similar adhesion

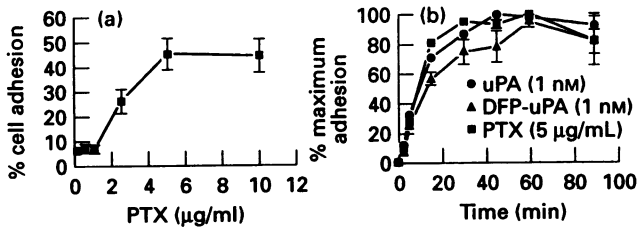

Figure 1. Adherent response of TGFβ1/D$_3$-differentiated U937 cells to PTX or uPA. (a) Concentration-dependent adhesion of U937 cells to PTX. Cells were labelled with [^{3}H]thymidine for 24 hr before they were primed with TGFβ1/D$_3$ for another 24 hr in medium containing 10% FBS. Primed U937 cells were then treated with various concentrations of PTX for 1 hr at 37°. Cell adhesion was expressed as percent of total c.p.m. from the control. (b) Time–course studies of adherent response of the U937 cells to PTX (5 μg/ml), uPA (1 nM), or DFP-uPA (1 nM). Results were expressed as percent of maximum adhesion in response to each stimulant. Each value represents mean ± SEM of three experiments performed in triplicate.

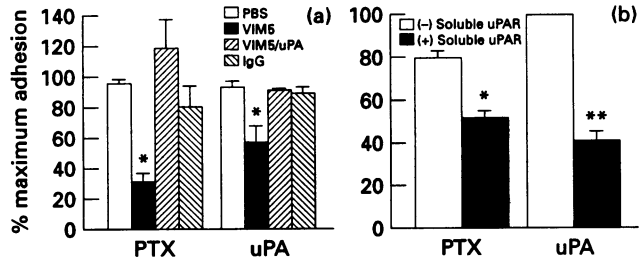

Figure 3. The role of uPAR in PTX- or uPA-induced differentiated U937 cell adhesion. (a) Effect of VIM5, a mAb (diluted 1 : 20) directed against the binding domain of the uPAR, on PTX- or uPA-induced U937 cell adhesion in serum-containing cultures. A concentration of 1 nM uPA was used to induce adhesion, whereas uPA was added to the VIM5 antibody where indicated at a final concentration of 50 nM. (b) Effect of conditioned medium containing soluble uPAR on PTX- or uPA-induced U937 cell adhesion. Each value represents mean ± SEM of three experiments performed in duplicate and is expressed as percent of maximum PTX- or uPA-induced adhesion. $*P < 0{\cdot}01$, $**P < 0{\cdot}001$, as compared to PTX or uPA control (ANOVA followed by Scheffe test).

kinetics were also observed for uPA or DFP-inactivated uPA, which are consistent with our previous findings.[10] Undifferentiated U937 cells did not exhibit an adherent response to PTX at concentration up to 10 μg/ml.

The role of uPAR in PTX-induced myeloid cell adhesion

Recent findings indicate that the uPAR is also a high affinity receptor for the matrix-like form of vitronectin.[15] Due to the similarities in the time–course of adherent responses of cells to uPA and PTX, we examined whether PTX-induced differentiated U937 cell adhesion was selective for vitronectin. Photographic data in Fig. 2 shows that indeed this is the case. TGF-β1/D$_3$-primed cells exhibited spontaneous adhesion to fibronectin and fibrinogen but little or no attachment to serum- or vitronectin-coated surfaces. PTX induced equivalent cellular adhesion to serum- and vitronectin-coated culture wells and decreased or completely blocked adhesion to fibronectin and fibrinogen, respectively. Therefore, we next examined whether PTX-induced U937 cell adhesion was mediated by uPAR. VIM5 (diluted 1 : 20, ~50 μg/ml), a mAb raised against the binding domain of uPAR substantially blocked PTX-induced differentiated U937 cell adhesion, and, to a lesser extent, the uPA-induced myeloid cell adhesion (Fig. 3a). In order to compete with the antibody for binding to uPAR, addition of excess amounts of uPA completely reversed the inhibition by VIM5; whereas a control purified mouse ascites IgG did not affect either of the adherent responses. When the cell adhesion assay was conducted in conditioned medium containing soluble uPAR, both the uPA- and PTX-induced myeloid cell adhesion was substantially reduced as compared to in the same medium lacking the soluble uPAR (Fig. 3b). Taken together, these observations indicate that PTX-induced myeloid cell adhesion is uPAR-dependent.

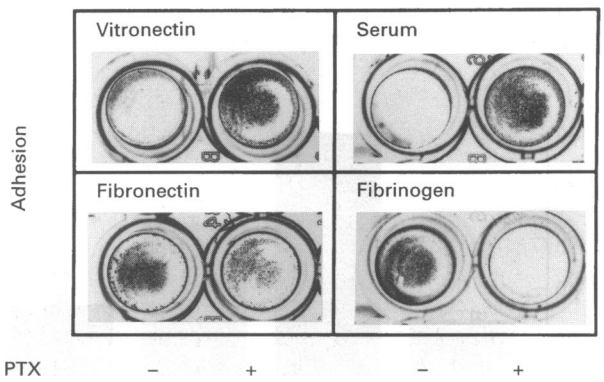

Figure 2. PTX induces TGFβ1/D$_3$-primed U937 cell adhesion selectively to vitronectin. Photograph of cell adhesion to the various extracellular matrices in the presence and absence of PTX (5 μg/ml). PTX-induced U937 cell adhesion to vitronectin-, fibronectin-, or fibrinogen-coated wells as compared to in serum. Aliquots of 100 μl of vitronectin (5 μg/ml), fibronectin (20 μg/ml), or fibrinogen (100 μg/ml) were added to 96-well plates and were allowed to coat the wells overnight at 4°. After blocking the coated wells with 1% bovine serum albumin (BSA) for 2 hr at 37°, adhesion assays were performed as described.

Role of B-subunit in PTX-induced myeloid cell adhesion

We next considered the mechanism by which PTX induced uPAR-dependent adhesion. The rapid stimulation of U937 cell adhesion suggested that the ADP-ribosyltransferase activity of PTX may not be required for this function. Our G-protein ribosylation experiment according to the method of Liang and Galper,[19] confirmed that indeed this is the case (data not shown). This would be similar to the mitogenic activity of PTX in T lymphocytes in which only the B-subunit of PTX (free of ADP-ribosyltransferase activity) is required.[4–8] We therefore used purified PTX B-subunit in the cell adhesion assay. Primed U937 cells became adherent in response to 5 μg/ml PTX B-subunit (Fig. 4) in a manner identical to the holotoxin. The adhesion was prevented by 10 mM ethylenediamine tetraacetic acid (EDTA) suggesting that the induced adhesion is divalent cation-dependent. Our recent finding showed that uPAR-dependent adhesion in these cells appears to be EDTA insensitive.[14] Therefore, EDTA could be acting at the level of PTX binding as well as at some downstream site in the adhesion process. Cholera toxin, another classical A-B subunit exotoxin,[1,2]

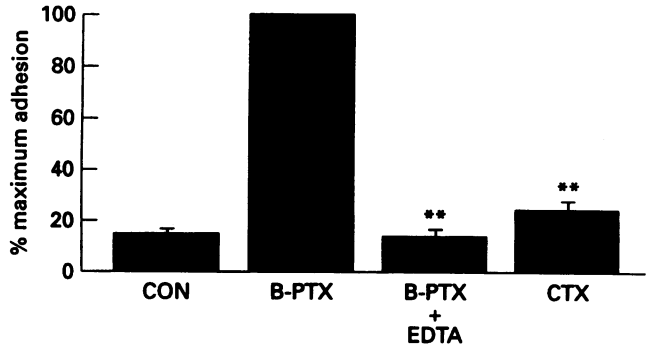

Figure 4. Adherent response of TGFβ1/D_3-differentiated U937 cells to purified B-subunit of PTX (B-PTX, 5 μg/ml) with and without 10 mM EDTA, or to cholera toxin (CTX, 100 μg/ml). Adhesion assays were performed as described. Each value represents mean ± SEM of three experiments performed in triplicate and is expressed as per cent of maximum adhesion by PTX B-subunit. $^{**}P < 0.001$, as compared to the PTX B-subunit control (ANOVA followed by Scheffe test).

at concentration up to 100 μg/ml, did not promote cells adhesion, suggesting that induction of myeloid cell adhesion was quite specific for PTX.

Effect of PTX on Ca^{2+} signalling

Having demonstrated that PTX rapidly induces myeloid cell adhesion, we investigated intracellular signalling events that occurred briefly following PTX treatment. Since PTX rapidly stimulates an increase in $[Ca^{2+}]_i$ in T cells,[4,5] we sought to determine whether it had a similar effect in U937 cells. PTX (5 μg/ml) triggered an immediate increase in $[Ca^{2+}]_i$ from TGF-β1/D_3-treated, but not from uninduced, U937 cells (Fig. 5a). This activity was also PTX concentration-dependent (Fig. 5b). The threshold concentration for PTX to elicit a Ca^{2+} spike was about 0·5 μg/ml, and maximum increase in $[Ca^{2+}]_i$, approximately 200% higher than the basal level, was attained at 5 μg/ml PTX, which closely approximate the concentration–response curve for induction of adhesion (Fig. 1a). When 3 mM EGTA, a selective Ca^{2+} chelator, was added to the cell suspension prior to PTX treatment, the induced rise in $[Ca^{2+}]_i$ concentration was attenuated by 50%, suggesting that the PTX-induced Ca^{2+} surge is contributed by both intracellular Ca^{2+} release and extracellular Ca^{2+} influx. DFP-uPA, which has been shown to promote myeloid cells adhesion, did not cause any changes in $[Ca^{2+}]_i$ (Fig. 5a). This indicated that uPA-induced cell adhesion did not involve Ca^{2+} as a signal, whereas, PTX, upon binding to certain cell surface molecule, induced $[Ca^{2+}]_i$ elevation which might eventually lead to myeloid cell adhesion.

Effect of Ca^{2+} chelator on PTX-induced myeloid cell adhesion

In an attempt to determine if PTX-induced rapid Ca^{2+} signalling in primed U937 cells actually mediated the subsequent adhesion response, we examined the inhibitory effects of BAPTA/AM, a selective membrane-permeant Ca^{2+} chelator, on PTX-induced myeloid cell adhesion. BAPTA/AM inhibited PTX-induced differentiated U937 cells adhesion in a concentration-dependent manner (Fig. 6). At 25 μM, BAPTA/AM completely prevented myeloid cell adhesion by PTX. This observation confirms a major role for $[Ca^{2+}]_i$ elevation in mediating PTX-induced myeloid cell adhesion. Indeed, Ca^{2+} ionophore A23187 (0·1–100 μM) alone, promoted adhesion in primed U937 cells that was 82% ($n = 2$ experiments performed in duplicate) of adhesion produced by 5 μg/ml PTX.

Role of Mac-1 in PTX-induced myeloid cell adhesion

In addition to inducing primed U937 cell adhesion to vitronectin- or serum-coated plates (Fig. 2), PTX blocked spontaneous cellular adhesion to fibrinogen, and, to a lesser extent, to fibronectin. Our flow cytometry studies revealed that preincubation of U937 cells with TGF-β1/D_3 for 24 hr increased cell surface expression of the Mac-1 integrin receptor by 2·6-fold (mean fluorescence intensity from 27·5 ± 3·8 for U937 cells to 70·9 ± 10·6 for stimulated U937 cells, $P < 0.05$, $n = 3$) and addition of PTX to these primed U937 cells did not alter Mac-1 (CD11b) expression (59·03 ± 9·09, $n = 3$).

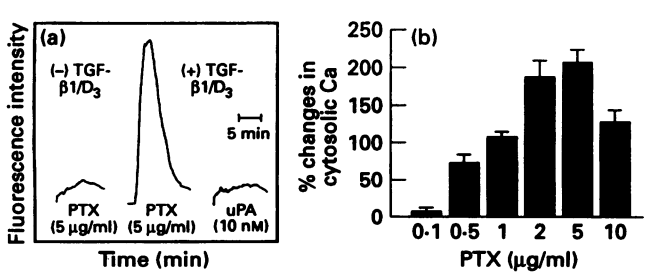

Figure 5. Effect of PTX on Ca^{2+} signalling. Differentiated U937 cells were loaded with Fluo-3/AM (10 μM) for 1 hr at 37° and were washed before aliquots of cell suspension were transferred to cuvettes and treated with PTX or uPA. Changes in Ca^{2+}-specific fluorescence were measured by a spectrofluorometer. (a) Representative tracings of changes in $[Ca^{2+}]_i$ as reflected by changes in fluorescence intensity from control and differentiated U937 cells treated with PTX (5 μg/ml) or uPA (10 nM). (b) Concentration-dependent effect of PTX on changes in $[Ca^{2+}]_i$ in differentiated U937 cells. Each value represents mean ± SEM of 3–4 experiments.

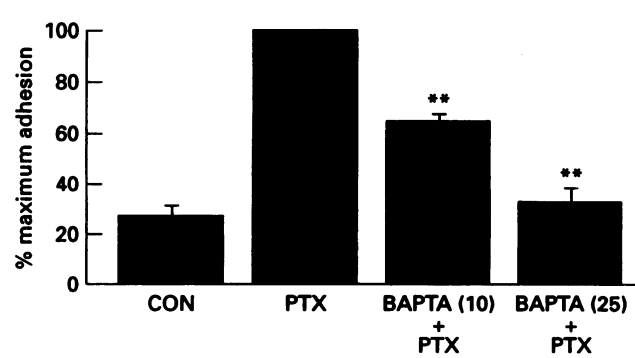

Figure 6. Effects of intracellular Ca^{2+} chelator BAPTA/AM on PTX-induced primed U937 cell adhesion. U937 cells were loaded with BAPTA/AM (10 or 25 μM) for 1 hr at 37° and adhesion assays were performed as described. Each value represents mean ± SEM of three experiments in duplicate and is expressed as per cent of maximum adhesion by PTX. $^{*}P < 0.05$, $^{**}P < 0.001$, as compared to the PTX control (ANOVA followed by Scheffe test).

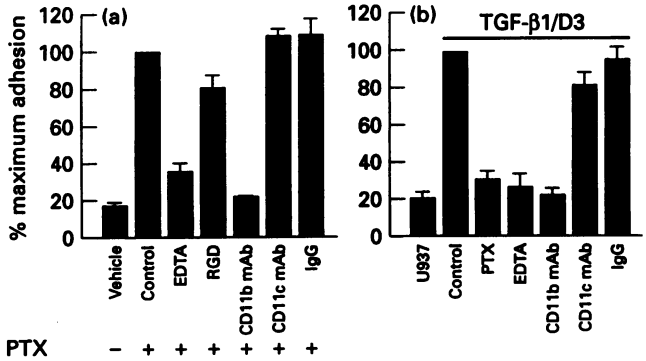

Figure 7. Role of Mac-1 in PTX-induced myeloid cell adhesion. (a) Effects of various mAbs (diluted 1 : 50), RGD-blocking peptide (1 mM), and 10 mM EDTA on PTX-induced U937 cell adhesion in serum-containing cultures. Each value represents mean ± SEM of 3–12 experiments performed in duplicate. (b) Effects of PTX (5 μg/ml), EDTA (10 mM), and various mAbs (diluted 1 : 50) on U937 cell binding to PTX-coated wells. In these experiments wells were pre-coated with PTX (5 μg/ml), blocked with albumin, and then adhesion assays performed under serum-free conditions. Each value is mean ± SEM of 2–7 experiments performed in duplicate.

Therefore we considered the possibility that PTX might interact with Mac-1.

A mouse mAb (2LMP19c), directed against the I domain of the CD11b of Mac-1 and known to block the binding of fibrinogen, iC3b and intercellular adhesion molecule (ICAM)-1 to Mac-1,[20,21] completely prevented PTX-induced primed U937 cell adhesion (Fig. 7a). Based on this observation, we repeated the measurements of $[Ca^{2+}]_i$ following PTX stimulation of primed U937 cells in the presence of 2LPM19c. The CD11b blocking antibody also completely abrogated the PTX-induced rise in $[Ca^{2+}]_i$ (Fig. 8). 2LPM19c mAb alone did not cause any changes in $[Ca^{2+}]_i$. Thus, we hypothesized that PTX interacted directly with the Mac-1 receptor and explored the ability of primed U937 cells to adhere to PTX-coated plate. Figure 7b shows that primed U937 cells bind to PTX-coated plates and that 2LMP19c prevented this binding. MAb against the α-subunit of gp150,95 (CD11c/CD18), mouse ascites IgG_1 and

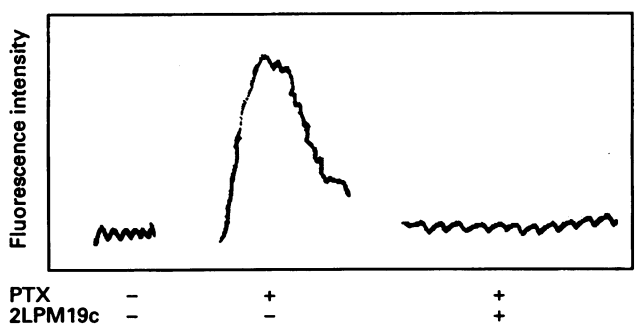

Figure 8. Effect of anti-CD11b mAb, 2LPM19c, on PTX-induced $[Ca^{2+}]_i$ changes in TGFβ1/D_3-differentiated U937 cells. Cells were loaded with Fluo-3/AM (10 μM) for 1 hr at 37° and were washed before aliquots of cell suspension were transferred to cuvettes. Cells were pre-treated with 2LPM19c (diluted 1 : 30) for 10 min before PTX (5 μg/ml) addition. Changes of Ca^{2+}-specific fluorescence were measured by a spectrofluorometer.

RGD-blocking peptide did not demonstrate any inhibitory effect (Fig. 7a). It was previously shown that RGD sequences on certain ligands are important for Mac-1 integrin binding. However, more recent studies have demonstrated that ICAM-1, iC3b, and fibrinogen do not use RGD fragment to bind to Mac-1.[22,23] Our data showed that PTX-induced myeloid cell adhesion was RGD-resistant as well. EDTA prevented the binding of differentiated U937 cells to PTX-coated plates (Fig. 7b), consistent with a divalent cation-dependent mechanism of the attachment of the toxin to myeloid cells[24,25] and of Mac-1 receptor activation.[9] In line with these findings, fibrinogen (1 mM), an endogenous ligand for Mac-1, also promoted adhesion of primed U937 cells equivalent to that by PTX (fibrinogen : PTX = 112% : 100%, $n = 5$ experiments in duplicate) and the adhesion was substantially reduced by 10 mM EDTA to only 10·1 ± 4·3% ($n = 4$ experiments in duplicate, $P < 0{\cdot}001$) of maximum fibrinogen-induced adhesion.

DISCUSSION

Our previous studies showed that uPA induces TGF-β1/D_3-primed myeloid cell adhesion to serum- and vitronectin-coated surfaces.[10,14] In the course of investigating potential G-protein involvement in uPA-induced myeloid cell adhesion, we found that PTX was able to produce adherent response of TGF-β1/D_3-primed, but not uninduced, human monoblastic U937 cells analogous to uPA. The adherent responses of TGF-β1/D_3-stimulated U937 cells by PTX is clearly independent of ADP-ribosylation of the Gi protein because purified B-subunit of PTX (free of ADP-ribosyltransferase activity) can reproduce the same adherent responses as induced by the holotoxin, suggesting that PTX-induced myeloid cell adhesion is primarily associated with the binding property of the B-subunit.[1,2]

Although there is ample evidence to substantiate the rapid stimulatory effect of the B-subunit of PTX in a variety of cell types,[4–8] the specific cell surface receptor for PTX has not been clearly described. Several lines of evidence point to Mac-1 as a potential cellular binding site for PTX. Recent findings revealed that the PTX B-subunit bears amino acid sequence homology to eukaryotic carbohydrate recognition domain of the selectin family.[24,25] Selectins comprise a family of cell surface glycoproteins exhibiting specific binding to sialyl Lewis x and sialyl Lewis a determinants on leucocytes and endothelial cells.[26] PTX has been shown to block neutrophil adherence to selectin-coated substrates, and antibodies raised against Lewis x and Lewis a determinants prevent the binding of PTX to human macrophages.[27,28] It has been demonstrated that the Lewis x determinant (CD15) is expressed on the Mac-1 integrin receptor,[27–29] and mAb against CD11b, but not CD11c, completely blocked the anti-Lewis x mAb-induced neutrophil homoaggregation.[29] The present study showed that 2LPM19c, a mAb raised against the α subunit of Mac-1 and mapped to its I domain, completely prevented PTX-induced myeloid cell adhesion in serum and blocked TGF-β1/D_3-stimulated U937 cell adherence to PTX-coated plates. In addition, PTX substantially inhibited the spontaneous adhesion of primed U937 cells to fibrinogen, an endogenous Mac-1 ligand.[9] Moreover, fibrinogen alone produced primed U937 cell adhesion in serum-containing cultures. Taken together, our observation suggests that PTX may bind to Mac-1 leading to myeloid cell adhesion.

That a transient, rapid rise in $[Ca^{2+}]_i$ largely underlies the pro-adhesive effect of PTX is consistent with prior works examining the signalling pathway of PTX B-subunit. In human T lymphocytes, PTX has been shown to trigger a rapid rise in $[Ca^{2+}]_i$, diacylglycerol and IP_3, and to activate protein kinase C and tyrosine kinase, which may eventually contribute to the mitogenic response of T cells.[4–6] In human platelets, PTX induces rapid accumulation of IP_3 and phosphatidic acid, leading to ensuing platelet aggregation.[8] All these early signal transduction events can be reproduced by either purified B-subunit moiety or *N*-ethylmaleimide-modified PTX in which the ADP-ribosyltransferase is inactivated.[4–6] Our data also reveal that PTX triggered a rapid transient elevation of $[Ca^{2+}]_i$ in TGF-β1/D_3-primed, but not uninduced, U937 cells. Incubation with BAPTA/AM, a selective membrane-permeant Ca^{2+} chelator, inhibited PTX-induced primed U937 cell adhesion clearly confirming the role of cytosolic Ca^{2+} rise in the subsequent cell adhesion. Moreover, the Ca^{2+} ionophore, A23187, promoted primed U937 cell adhesion comparable to PTX. However, the relative contribution of intracellular Ca^{2+} release and extracellular Ca^{2+} entry to the adherent response remains to be determined. Nevertheless, Bradford *et al.*[30] recently reported that vitamin D_3 enhances the transcription and expression of IP_3 receptor gene in HL-60 cells resulting in greater Ca^{2+} mobilization from the intracellular store induced by IP_3. These findings enable us to speculate that intracellular Ca^{2+} release might play a major role in PTX-induced myeloid cell adhesion. Moreover, PTX-induced differentiated U937 cell adhesion to serum- or vitronectin-coated wells was totally abolished when experiments were conducted at 4° (data not shown), indicating that the induced adherent response requires normal cellular metabolic processes, such as Ca^{2+} mobilization.

Mac-1 receptor–ligand occupancy has been shown to initiate transient $[Ca^{2+}]_i$ elevation,[31,32] which further supports our contention that PTX may bind to Mac-1 to induce $[Ca^{2+}]_i$ changes. The finding that 2LPM19c, a mAb to CD11b, prevented both the $[Ca^{2+}]_i$ surge and the subsequent adhesion suggests that the $[Ca^{2+}]_i$ rise is causally linked to the increased adhesiveness of the cell. In contrast, 10 nM uPA, which has been shown to produce myeloid cell adhesion to vitronectin- or serum-coated plates,[10,14] did not elicit any changes in $[Ca^{2+}]_i$, suggesting different intracellular signalling mechanisms mediating adherent responses by PTX and uPA.

We recently identified uPAR as a novel class of adhesion receptor with vitronectin specificity.[15] Because of these recent findings we sought to determine if the myeloid cell adhesion produced by PTX is uPAR-dependent. The ability of antibodies to uPAR (VIM5) and excess soluble uPAR itself, to substantially reduce the proadhesive effects of PTX on adhesion indicates that this is indeed the case. This is consistent with vitronectin binding data in these cells which show uPAR to be their major vitronectin receptor.[14] On the other hand, $\alpha_V\beta_5$ integrin has been shown to bind selectively to vitronectin as well.[33] Our flow cytometry study identified no expression of this cell surface adhesion molecule on either uninduced or TGF-β1/D_3-primed U937 cells (data not shown), indicating that $\alpha_V\beta_5$ is not responsible for the PTX-induced cell adhesion to vitronectin. However, since both the VIM5 and soluble uPAR did not totally block the PTX-induced myeloid cell adhesion, involvement of potential uPAR-independent adhesion mechanisms cannot be excluded. The molecular mechanisms, subsequent to Ca^{2+} mobilization, such as assembly/stabilization of cytoskeletal elements, by which PTX promotes uPAR-mediated adhesion remain to be defined.

Since alveolar macrophage is the major host defence to *B. pertussis* infection, it becomes very important to understand the interaction between PTX and macrophage at the receptor, signal transduction, and functional levels. We propose that the PTX B-subunit may bind to Mac-1 integrin of myeloid cells to rapidly elevate $[Ca^{2+}]_i$ leading to activation of uPAR for adherence to vitronectin. Mac-1 has recently been demonstrated to be physically associated with uPAR in neutrophils, raising the possibility of co-operation between these receptors.[34,35] Further experiments to isolate the binding site(s) of PTX in myelomonocytic cells and to elucidate the signalling sequences leading to uPAR activation are deemed warranted.

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REFERENCES

1. Tamura M., Nogimori K., Murai S. *et al.* (1982) Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. *Biochemistry* **21,** 5516.
2. Nicosia A., Perguni M., Franzini C. *et al.* (1986) Cloning and sequencing of the pertussis toxin genes: operon structure and gene duplication. *Proc Natl Acad Sci USA* **83,** 4631.
3. Gierschik P. (1992) ADP-ribosylation of signal-transducing guanine nucleotide-binding proteins by pertussis toxin. *Curr Top Microbiol Immunol* **175,** 69.
4. Rosoff P.M., Walker R. & Winberry L. (1987) Pertussis toxin triggers rapid second messenger production in human T lymphocytes. *J Immunol* **139,** 2419.
5. Rosoff P.M. & Mohan C. (1992) Unidirectional, heterologous desensitization of the pertussis toxin receptor by the CD3/TCR complex. *J Immunol* **149,** 3191.
6. Strand C.F. & Carchman R.A. (1987) Human T lymphocyte mitogenesis in response to the B-oligomer of pertussis toxin is associated with an early elevation in cytosolic calcium concentrations. *FEBS Lett* **225,** 16.
7. Zhang X.M., Berland R. & Rosoff P.M. (1995) Differential regulation of accessory mitogenic signaling receptors by the T cell antigen receptor. *Molec Immunol* **32,** 323.
8. Banga H.S., Walker R.K., Winberry L.K. & Rittenhouse S.E. (1987) Pertussis toxin can activate human platelets. Comparative effects of holotoxin and its ADP-ribosylating S_1 subunit. *J Biol Chem* **262,** 14871.
9. Hynes R.O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69,** 11.
10. Waltz D.A., Sailor L.Z. & Chapman H.A. (1993) Cytokines induced urokinase-dependent adhesion of human myeloid cells. A

regulatory role for plasminogen activator inhibitors. *J Clin Invest* **91,** 1541.

11. HARRIS P. & RALPH P. (1985) Human leukemic models of myelomonocytic development: A review of the HL-60 and U937 cell lines. *J Leuk Biol* **37,** 407.
12. MORIKAWA M., HARADA N., SOMA G.I. & YOSHIDA T. (1990) Transforming growth factor-β1 modulates the effect of 1_{α}, 25-dihydroxyvitamin D_3 on leukemic cells. *In Vitro Cell Dev Biol* **26,** 682.
13. IGNOTZ R.A., HEINO J. & MASSAGUE J. (1989) Regulation of cell adhesion receptors by transforming growth factor-β. Regulation of vitronectin receptor and LFA-1. *J Biol Chem* **264,** 389.
14. WALTZ D.A. & CHAPMAN H.A. (1994) Reversible cellular adhesion to vitronectin linked to urokinase receptor occupancy. *J Biol Chem* **269,** 14746.
15. WEI Y., WALTZ D.A., RAO N.K., DRUMMOND R., ROSENBERG S. & CHAPMAN H.A. (1994) Identification of the urokinase receptor as an adhesion receptor for vitronectin. *J Biol Chem* **269,** 32380.
16. RAO N.K., SHI G.P. & CHAPMAN H.A. (1995) Urokinase receptor is a multifunctional protein: Influence of receptor occupancy on macrophage gene expression. *J Clin Invest* **96,** 465.
17. YATOHGO T., IZUMI M., KASHIWAGI H. & HAYASKI M. (1988) Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct Funct* **13,** 281.
18. KAO J.P.Y., HAROOTUNIAN A.T. & TSIEN R.Y. (1989) Photochemically generated cytosolic calcium pulse and their detection by fluo-3. *J Biol Chem* **264,** 8179.
19. LIANG B.T. & GALPER J.B. (1988) Differential sensitivity of αo and αi to ADP-ribosylation by pertussis toxin in the intact cultured embryonic chick ventricular myocyte. *Biochem Pharmacol* **37,** 4549.
20. DIAMOND M.S. & SPRINGER T.A. (1993) A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen. *J Cell Biol* **120,** 545.
21. DIAMOND M.S., GARCIA-AGUILAR J., BICKFORD J.K., CORBI A.L. & SPRINGER T.A. (1993) The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. *J Cell Biol* **120,** 1031.
22. SIDLE-TANIGUCHI A. & ISENMAN D.E. (1992) Mutagenesis of the Arg-Gly-Asp triplet in human complement component C3 does not abolish binding of iC3b to the leukocyte integrin complement receptor type III (CR3, CD11b/CD18). *J Biol Chem* **267,** 635.
23. ALTIERI D.C., PLESCIA J. & PLOW E.F. (1993) The structural motif glycine 190-valine 202 of the fibrinogen gamma chain interacts with CD11b/CD18 integrin ($\alpha_m\beta_2$, Mac-1) and promotes leukocyte adhesion. *J Biol Chem* **268,** 1847.
24. ROZDZINSKI E., BURNETTE W.N., JONES T., MAR V. & TUOMANEN E. (1993) Prokaryotic peptides that block leukocyte adherence to selectins. *J Exp Med* **178,** 917.
25. SAUKKONEN K., BURNETTE W.N., MAR V.L., MASURE H.R. & TUOMANEN E.I. (1992) Pertussis toxin has eukaryotic-like carbohydrate recognition domains. *Proc Natl Acad Sci USA* **89,** 118.
26. LASKY L.A. (1992) Selectins: Interpreters of cell-specific carbohydrate information during inflammation. *Science* **258,** 964.
27. STOCK S.C., ALBRECHTSEN M. & KERR M.A. (1990) Expression of the CD15 differentiation antigen (3-fucosyl-*N*-acetyl-lactosamine, Le) on putative neutrophil adhesion molecules CR3 and NCA-160. *Biochem J* **268,** 275.
28. SKUBITZ K.M. & SNOOK II, R.W. (1987) Monoclonal antibodies that recognize lacto-*N*-fucopentose III (CD15) react with the adhesion-promoting glycoprotein family (LFA-1/HMAC-1/GP 150, 95) and CR1 on human neutrophils. *J Immunol* **139,** 1631.
29. STOCKL J., MAJDIC O., ROSENKRANZ A. *et al.* (1993) Monoclonal antibodies to the carbohydrate structure Lewis^x stimulate the adhesive activity of leukocyte integrin CD11b/CD18 (CR3, Mac-1, $\alpha_m\beta_2$) on human granulocytes. *J Leuk Biol* **53,** 541.
30. BRADFORD P.G., JIN Y. & HUI P. (1993) 1,25-Dihydroxyvitamin D_3 enhances the transcription and expression of the inositol trisphosphate receptor gene in HL-60 cells. *Mol Pharmacol* **44,** 292.
31. ALTIERI D.C., STAMNES S.J. & GAHMBERG C.G. (1992) Regulated Ca^{++} signalling through leukocyte CD11b/CD18 integrin. *Biochem J* **288,** 465.
32. NG-SIKORSKI J., ANDERSSON R., PATARROYO M. & ANDERSSON T. (1991) Calcium signaling capacity of the CD11b/CD18 integrin on human neutrophils. *Exp Cell Res* **195,** 504.
33. SMITH J.W., VESTAL D.J., IRWIN S.V., BURKE T.A. & CHERESH D.A. (1990) Purification and functional characterization of integrin $\alpha_V\beta_5$. An adhesion receptor for vitronectin. *J Biol Chem* **265,** 11008.
34. XUE W., KINDZELSKII A.L., TODD R.F. & PETTY H.R. (1994) Physical association of complement receptor type 3 and urokinase-type plasminogen activator receptor in neutrophil membranes. *J Immunol* **152,** 4630.
35. CAO D., MIZUKAMI I.F., GAMI-WAGNER B.A. *et al.* (1995) Human urokinase-type plasminogen activator primes neutrophils for superoxide anion release. Possible roles of complement receptor type 3 and calcium. *J Immunol* **154,** 1817.