

## Mechanisms of pertussis toxin-induced myelomonocytic cell adhesion: role of CD14 and urokinase receptor

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### SUMMARY

Pertussis toxin (PTX) has been shown previously to promote myelomonocytic cell adhesion in serum. The aim of the present study was to identify, using transforming growth factor- $\beta_1$  and 1,25-(OH) $_2$  vitamin D $_3$  (TGF- $\beta_1$ /D $_3$ )-primed U937 cells, the PTX-binding site(s) and the adhesion molecule(s) responsible for PTX-induced myelomonocytic cell adhesion. Monoclonal antibodies (mAbs) directed against CD14, CD11b, CD18 or urokinase receptor (uPAR) significantly inhibited PTX-induced primed U937 cell adhesion in serum in a concentration-dependent manner. However, only anti-CD14 and anti-CD18 mAbs were able to prevent the myeloid cells from binding to PTX-coated plates and significantly inhibited a PTX-induced rise of  $[Ca^{2+}]_i$  in primed U937 cells. A receptor-isolation study showed that biotinylated PTX recognized a 48 000-molecular weight protein in primed U937 cell lysates, which could be specifically blocked by excess unlabelled PTX or by anti-CD14 mAb. On the other hand, mAb directed against uPAR significantly blocked PTX-induced myeloid cell adhesion to serum and to immobilized vitronectin, a major extracellular matrix protein in serum. Taken together, our data suggest that PTX may bind to cell-surface CD14 to induce myelomonocytic cell adhesion to vitronectin in serum via uPAR activation, which may represent a pathogenetic mechanism for the respiratory tract infection induced by *Bordetella pertussis*.

### INTRODUCTION

Pertussis toxin (PTX) is a major virulence factor mediating the pathogenesis of respiratory tract infection induced by *Bordetella pertussis*<sup>1</sup> and is the only virulence component present in all types of acellular pertussis vaccine preparations.<sup>2</sup> PTX is a heterohexamer that is functionally divided into A and B subunits, analogous to cholera toxin. The A-protomer consists of a single polypeptide (S1) that possesses ADP-ribosyltransferase activity, and the B-oligomer confers binding specificity to the target cell membrane.<sup>3,4</sup>

Although *B. pertussis* infection is a well-recognized disease, the pathogenesis of the disease process is still poorly under-

stood. Upon prolonged incubation (at least 1–2 hr) with PTX, the A-protomer will be internalized by certain cells and ADP ribosylates the  $\alpha$ -subunit of the membrane-bound Gi-like protein, leading to blockade of certain transmembrane signalling process and eventually cellular intoxication.<sup>5</sup> In addition to its delayed inhibitory effect on the Gi protein, PTX has also been shown to elicit rapid responses (in minutes) in a variety of cell types,<sup>6</sup> which may have profound pathological effects as important as its ADP-ribosylation activity. All of these rapid cellular responses can be reproduced by the purified PTX B-oligomer alone, suggesting that occupation of the PTX-binding site(s) mediates these early cellular events.

Progress has been made regarding the binding properties of PTX. It has been shown that the S2 and S3 subunits of the B-oligomer possess a carbohydrate-recognition domain that could selectively bind to Lewis a (Le<sup>a</sup>) and Lewis x (Le<sup>x</sup>) determinants.<sup>7</sup> In separate receptor-binding studies, PTX was found to bind to a 165 000-molecular weight (MW) sialylated glycoconjugate on Chinese hamster ovary (CHO) cells,<sup>8</sup> to 43 000-MW and 70 000-MW cell-surface proteins on a Jurkat cell line,<sup>9–11</sup> and to 164 000-MW sialoglycoprotein Ib (GPIb), known to be activated by von Willebrand factor, on the platelet membrane.<sup>12</sup>

More recently, PTX holotoxin, as well as its binding subunit, B-oligomer, have been shown to block entry of

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Abbreviations:  $[Ca^{2+}]_i$ , intracellular calcium concentration; D $_3$ , 1,25-(OH) $_2$  vitamin D $_3$ ; FITC, fluorescein isothiocyanate; HIV, human immunodeficiency virus; mAb, monoclonal antibody; PTX, pertussis toxin; TGF- $\beta_1$ , transforming growth factor- $\beta_1$ ; uPAR, urokinase receptor.

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monotropic (R5) strains of human immunodeficiency virus-1 (HIV-1) in primary T lymphocytes. In addition, the PTX B-oligomer inhibited virus production in peripheral blood mononuclear cells infected with either R5 or X4 strains of HIV-1.<sup>13</sup> These findings suggested that the PTX B-oligomer deactivated a chemokine receptor (CCR5, a co-receptor for HIV-1) via binding to a yet-unknown PTX receptor on T cells and initiation of a series of cellular signalling events.<sup>13,14</sup> There is an unequivocal need to identify the cellular binding site(s) for PTX and to ascertain the B-oligomer-mediated cellular signalling events.

As monocytes/macrophages are the major host defence against *B. pertussis* infection and a major target for HIV-1, it becomes very important to identify the binding site(s) for PTX on myelomonocytic cells and to understand the functional consequences upon receptor occupation. Our recent study showed that PTX holotoxin, as well as PTX B-oligomer, induced a rapid adherent response of myelomonocytic cells to serum via urokinase receptor (uPAR), a high-affinity receptor for vitronectin.<sup>15,16</sup> The present study was undertaken to explore the interaction between PTX and myelomonocytic cells at the receptor- and adherent-response levels using transforming growth factor- $\beta_1/1,25\text{-}(\text{OH})_2$  vitamin D<sub>3</sub> (TGF- $\beta_1/\text{D}_3$ )-primed U937 cells. Results obtained from the receptor-isolation and cell-adhesion studies indicate that CD14 is probably a binding site for PTX on myelomonocytic cells. In addition, using monoclonal antibodies (mAbs) against the binding domain of uPAR, our data confirmed that PTX induced myeloid cell adhesion to vitronectin via activation of uPAR.

## MATERIALS AND METHODS

### Materials

The human monoblastic leukaemic U937 cell line was obtained from the American Type Culture Collection (Rockville, MD). TGF- $\beta_1$  was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and D<sub>3</sub> was a gift of Dr M. Manganel and Dr E. M. Gutknecht (Hoffman-LaRoche Ltd., Basel, Switzerland). RPMI-1640, methionine-free RPMI-1640, fetal bovine serum (FBS), HEPES, penicillin G/streptomycin, Geimsa stain and mouse mAb against  $\alpha_v$  integrin (clone VNR147) were from Life Technologies, Inc. (Gaithersburg, MD). Purified PTX was a gift of Dr P. Licari and Dr C. Nesman (Massachusetts Public Health Biologic Laboratories, Boston, MA). Purified PTX B-oligomer was obtained from Research Biochemicals International (Natick, MA). Mouse mAbs against CD14 (clone TÜK4), CD11b (clone 2LPM19c), CD11c (clone KB90), CD18 (clone MHM23) and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse F(ab')<sub>2</sub> fragments were obtained from DAKO (Glostrup, Denmark). Paraformaldehyde was obtained from Merck KGaA (Darmstadt, Germany). Mouse immunoglobulin G (IgG) and Fluo-3/AM were purchased from Calbiochem-Novabiochem (La Jolla, CA). Mouse mAb against domain II of uPAR was purchased from American Diagnostica, Inc. (Greenwich, CN). Vitronectin was obtained from Becton-Dickinson Labware (Bedford, MA). Bovine serum albumin (BSA), probenecid, manganese chloride (MnCl<sub>2</sub>) and ethylene glycol-bis( $\beta$ -aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) were from Sigma Chemical Co. (St. Louis, MO). Sulpho-NHS-LC-biotin and streptavidin-

agarose were purchased from Pierce Chemical Co. (Rockford, IL). [<sup>35</sup>S]Methionine was purchased from Amersham Pharmacia Biotech UK Ltd. (Little Chalfont, Bucks., UK).

### Cell culture

The human monoblastic leukaemic U937 cell line was maintained in RPMI-1640 supplemented to 10% (v/v) with FBS, 20 mM HEPES (pH 7.4), 100 U/ml of penicillin G and 100  $\mu\text{g}/\text{ml}$  of streptomycin, at 37° in a humidified incubator in an atmosphere of 5% CO<sub>2</sub>. Before any experimentation, cells were seeded at 10<sup>6</sup>/ml and differentiated to a monocyte/macrophage phenotype with 1 ng/ml of TGF- $\beta_1$  and 50 nM D<sub>3</sub> (TGF- $\beta_1/\text{D}_3$ ) for 48 hr. Differentiated U937 cells were determined by morphological changes observed using microscopy and by cell-surface expression of the monocytic differentiation antigen, CD14, and other macrophage-related integrins such as CD11b, CD11c and CD18. Cells without TGF- $\beta_1/\text{D}_3$  pretreatment were used as controls.

### Flow cytometry

Cells ( $5 \times 10^5$ ) were suspended in 200  $\mu\text{l}$  of RPMI-1640 containing 2.5% FBS, and the Fc receptors were blocked by incubation with human serum, at a final concentration of 1%, for 15 min at 4°. Mouse mAbs directed against CD14, CD11b, CD11c and CD18 were then incubated with the cell suspensions for 30 min on ice. The cells were washed three times in RPMI-1640 containing 2.5% FBS and then resuspended in 200  $\mu\text{l}$  of the same medium containing FITC-conjugated rabbit anti-mouse F(ab')<sub>2</sub> fragments and incubated for a further 30 min on ice. Cells were washed three times in phosphate-buffered saline (PBS) containing 0.5% BSA and then fixed in 200  $\mu\text{l}$  of prechilled 0.5% paraformaldehyde in PBS for 15 min. The paraformaldehyde was washed off and the cells were resuspended in PBS at a concentration of 10<sup>6</sup> cells/ml. The mean fluorescence value of the cell population was determined by measuring the fluorescence intensity of 10 000 cells using a Coulter Epics Elite Esp Flow Cytometry System (Coulter, Fullerton, CA). Excitation wavelength was set at 488 nm, and emission wavelength was at 525 nm. Non-specific fluorescence was determined on cells incubated with mouse IgG, i.e. irrelevant antigen specificity. Background was determined on cells probed with only the secondary antibody.

### Adhesion assays

TGF- $\beta_1/\text{D}_3$ -primed U937 cells were suspended at a concentration of 10<sup>6</sup> cells/ml in RPMI-1640 containing 10% FBS. Cells were treated with PTX or purified PTX B-oligomer in the presence or absence of various mAbs. Aliquots (100  $\mu\text{l}$ ) of treated cells were then placed in 96-well microtitre plates in duplicate. PTX-induced cell adhesion in serum was allowed to take place for 1 hr at 37°. Non-adherent cells were flicked out and 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 microplate reader (Tecan, Grödig, Austria). Data obtained from PTX-induced cell adhesion in the absence of mAb treatment were considered as the 100% control adherent response, and the effects of mAbs were expressed as a percentage of the control adherent response.

To determine the interaction between PTX and the cell-surface binding site(s) on primed U937 cells, we examined the direct binding of primed U937 cells to immobilized PTX. One-

**Table 1.** Expression of monocyte-differentiation antigens on U937 cells before and after treatment with transforming growth factor- $\beta_1$  and 1,25-(OH) $_2$  vitamin D $_3$  (TGF- $\beta_1$ /D $_3$ )

	Mean fluorescence intensities	
	Control	TGF- $\beta_1$ /D $_3$
Background (FITC)	3.0 ± 0.4	3.2 ± 0.8
IgG	2.7 ± 0.2	2.9 ± 0.3
CD11c	5.3 ± 0.2	6.6 ± 0.2*
CD14	3.0 ± 0.2	15.5 ± 2.0*
CD11b	13.5 ± 0.8	101.3 ± 12.4*
CD18	115.9 ± 3.3	296.7 ± 11.4*

\* $P < 0.05$  compared with control U937 cells.

Each value represents mean ± SEM of three to four experiments. The mean fluorescence value of the cell population was determined by measuring the fluorescence intensity of 10 000 cells.

FITC, fluorescein isothiocyanate.

hundred microlitre aliquots of PTX (10  $\mu$ g/ml) in PBS were added to 96-well microtitre plate and were allowed to coat the wells overnight at 4°. After blocking the coated wells with 1% BSA for 2 hr at 37°, the experiments were then performed, as described above, in the absence of FBS and added PTX.

To confirm that PTX-induced myeloid cell adhesion to vitronectin in serum is mediated by uPAR, we examined the adherent response in the presence and absence of anti-uPAR mAb and anti- $\alpha_v$  mAb. One-hundred microlitre aliquots of vitronectin (5  $\mu$ g/ml) in PBS were added to 96-well microtitre plates and were allowed to coat the wells overnight at 4°. After blocking the coated wells with 1% BSA for 2 hr at 37°, the experiments were performed (as described above) in the absence of FBS.

#### Intracellular Ca $^{2+}$ measurement

Cells (10 $^6$ /ml) were loaded with the intracellular calcium concentration ([Ca $^{2+}$ ] $_i$ ) indicator dye Fluo-3/AM (10  $\mu$ M) for 1 hr at 37° in RPMI-1640 and then washed three times with a physiological buffer (140 mM NaCl, 4 mM KCl, 1 mM KH $_2$ PO $_4$ , 1.8 mM CaCl $_2$ , 0.8 mM MgCl $_2$ , 10 mM glucose and 0.1% BSA). Cells were then resuspended in 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  $\mu$ M was added to the cell suspension. After incubation with different mAbs for 30 min, a 40- $\mu$ l aliquot of cell suspension was transferred to a cuvette containing 2 ml of physiological buffer and then challenged with PTX (10  $\mu$ g/ml). (All procedures in the cuvette were performed with continuous stirring and at a constant temperature of 37°.) Ca $^{2+}$ -specific fluorescence was measured using a spectrofluorometer (Shimadzu, Tokyo, Japan), with excitation wavelength set at 506 nm and emission wavelength set at 526 nm. Maximum fluorescence value was achieved by the addition of 200 mM KCl and 0.5% Triton-X-100, and minimum fluorescence value was attained by the addition of 20 mM EGTA and 25 mM MnCl $_2$ . A  $K_d$  of 320 nM for Fluo-3 was used to calculate [Ca $^{2+}$ ] $_i$ , as described previously.<sup>17</sup>

#### Biotinylation of PTX

Biotinylation of PTX was performed according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL). PTX was dialysed against 50 mM NaHCO $_3$  (pH 8.0) at 4°

overnight and concentrated to  $\approx$  1 mg/ml using microcentrifuge filters (Millipore Co., Bedford, MA). The concentrated PTX was then incubated (at room temperature for 40 min with gentle rotation) with 1 mg/ml of sulpho-NHS-LC-biotin, at a molar ratio of 1 : 50 (PTX : biotin). The biotinylation reaction was stopped by dialysing the biotinylated PTX overnight at 4° against 25 mM Tris (pH 8.0), 25 mM glycine and 0.5 M NaCl. The biotinylated PTX was stored at -20° until use.

#### [ $^{35}$ S]Methionine labelling and receptor isolation

U937 cells (10 $^6$ /ml) were first primed with TGF- $\beta_1$ /D $_3$  in RPMI-1640, containing 10% FBS, for 24 hr and then incubated in methionine-free RPMI-1640 containing 10% FBS and 100  $\mu$ Ci/ml of [ $^{35}$ S]methionine (specific activity > 1000 Ci/mmol) for a further 24 hr. The radiolabelled cells were spun down at 800 g and washed four times with PBS. The cell aggregates were then incubated in lysis buffer [50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1% Triton-X-100, 2 mM phenylmethylsulphonyl fluoride (PMSF), 10  $\mu$ g/ml of aprotinin, 50  $\mu$ g/ml of benzamide, and 5  $\mu$ g/ml of pepstatin, leupeptin and antipain] for 1 hr at 4° with gentle shaking. The lysate was centrifuged for 20 min at 24 000 g and the supernatant was precleared by incubation with streptavidin-agarose (1  $\mu$ l/100  $\mu$ l of lysate) for 30 min at 4° with gentle rotation. After centrifugation, the supernatant was incubated with biotinylated PTX (10  $\mu$ g/10 $^6$  cells) in the presence or absence of excess unlabelled PTX (40  $\mu$ g/10 $^6$  cells) or different mAbs for 1 hr at room temperature. The biotinylated PTX:receptor complex was then precipitated by streptavidin-agarose, at a ratio of 1  $\mu$ l/ $\mu$ g of biotinylated PTX,<sup>18</sup> for 1.5 hr at 4°. After centrifugation at 800 g for 1 min at 4°, the pellet was washed four times with PBS containing 0.1% Nonidet P-40 (NP-40) and 100  $\mu$ g/ml of PMSF. The bound receptors for PTX were then eluted with 0.1 M glycine-HCl (pH 2.8) for 30 min at 4°, and the collected supernatant was titrated to neutral pH with 0.25 M NaOH, mixed with 0.25 vol. of 5 $\times$  electrophoresis sample buffer, boiled for 5 min, and subjected to 4–20% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was dried and developed by autoradiography.

#### Data analysis

All data are presented as mean  $\pm$  SEM. Statistical differences in adherent responses between the controls and the treatment groups were analysed using analysis of variance (ANOVA) followed by the Student Newman-Keuls test. The critical level for significance was set at  $P < 0.05$ .

## RESULTS

### TGF- $\beta_1$ /D $_3$ -induced U937 cell differentiation

Human monoblastic leukaemic U937 cells differentiated to a monocyte/macrophage phenotype after TGF- $\beta_1$ /D $_3$  treatment for 48 hr. The expression of monocyte differentiation antigen, CD14, and other differentiation-related adhesion proteins, including CD11c, CD11b and CD18, was monitored (using flow cytometry) before and after TGF- $\beta_1$ /D $_3$  priming. In control U937 cells, the levels of CD14 and CD11c were as low as the background value whereas the expression of CD11b and CD18 was substantially higher. The rank order for adhesion protein expression in control U937 cells was

CD18 >> CD11b > CD11c > CD14. After priming with TGF- $\beta_1$ /D<sub>3</sub> for 48 hr, the mean fluorescence intensities for cell-surface expression of CD18, CD11b, CD11c and CD14 were significantly increased by 2.6-, 7.5-, 1.2- and 5.1-fold, respectively. The level of CD18 expressed on differentiated U937 cells was substantially higher than that of the other three antigens (Table 1).

#### PTX-induced myelomonocytic cell adhesion

Figure 1(A) shows that only TGF- $\beta_1$ /D<sub>3</sub>-primed (panel b), but not unprimed (panel a), U937 cells adhered to serum in response to PTX. Both PTX holotoxin and PTX B-oligomer were able to promote primed U937 cell adhesion to the same extent. Mouse mAbs directed against CD14, CD11b and CD18, but not against CD11c, significantly inhibited PTX-induced primed U937 cell adhesion by 72%, 94% and 97%, respectively (Fig. 1B). In addition, mAb against domain II of uPAR (CD87), the binding domain for vitronectin,<sup>16</sup> substantially reduced the cell adhesion by 84%. These results indicate that CD14, CD11b, CD18 and uPAR are involved in PTX-induced myeloid cell adhesion in serum.

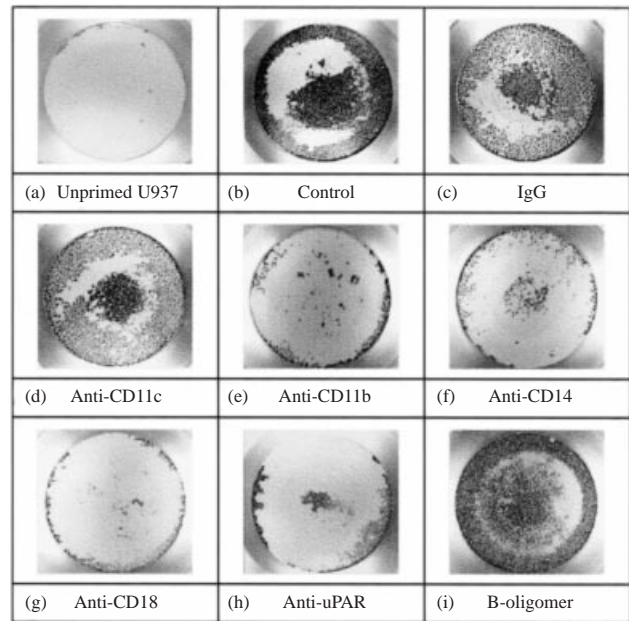
To further delineate which cell-surface proteins are the binding site(s) for PTX and which are responsible for the focal adhesion to serum, we examined the effects of increasing concentrations of mAbs on PTX-induced myelomonocytic cell adhesion in serum (for focal adhesion) and on cell binding to immobilized PTX (for PTX-binding sites). As shown in Fig. 2, anti-CD11c failed to block either PTX-induced cell adhesion to serum or cell binding to immobilized PTX at a concentration up to 3  $\mu$ g/ml. However, anti-CD14 inhibited, in a concentration-dependent manner, both the PTX-induced cell adhesion to serum (by up to 76%) and the cell binding to immobilized PTX (by up to 62%) at 3  $\mu$ g/ml, in a highly correlated manner ( $r=0.93$ ). In contrast, while the mAbs against CD11b, CD18 and uPAR dramatically abolished the PTX-induced cell adhesion to serum (focal adhesion) by 92%, 93% and 81%, respectively, at 0.5–1  $\mu$ g/ml, they failed to prevent primed U937 cells from binding to immobilized PTX (binding sites) at these same concentrations. At a higher concentration (2  $\mu$ g/ml), anti-CD18 mAb afforded a 48% inhibition on cell binding to PTX-coated plates.

uPAR has recently been shown to be a specific receptor for vitronectin, a major extracellular matrix protein present in serum.<sup>16,19</sup> We examined PTX-induced myelomonocytic cell adhesion to vitronectin-coated plates in the presence and absence of mAbs against uPAR. Figure 3 shows that PTX promoted primed, but not unprimed, U937 cell adhesion to vitronectin-coated plates. Mouse mAb directed against domain II of uPAR blocked PTX holotoxin- or PTX B-oligomer-induced cell adhesion to vitronectin by more than 90%. Anti- $\alpha_v$  mAb directed against other vitronectin receptors, such as  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$ ,<sup>20</sup> did not inhibit the PTX-induced cell adhesion to vitronectin-coated plates.

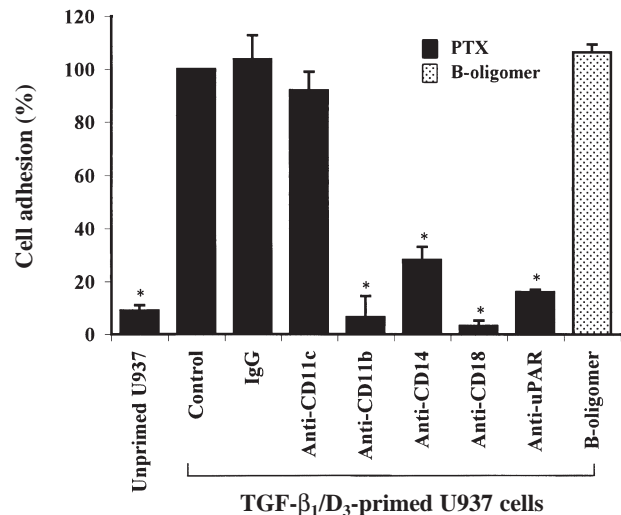
#### PTX-induced rise in [Ca<sup>2+</sup>]<sub>i</sub> in myelomonocytic cells

To functionally confirm the binding site(s) for PTX on myelomonocytic cells, we studied the PTX-induced rise of [Ca<sup>2+</sup>]<sub>i</sub> in the presence and absence of various mAbs. Figure 4(A) displays representative tracings of the changes in

(A)

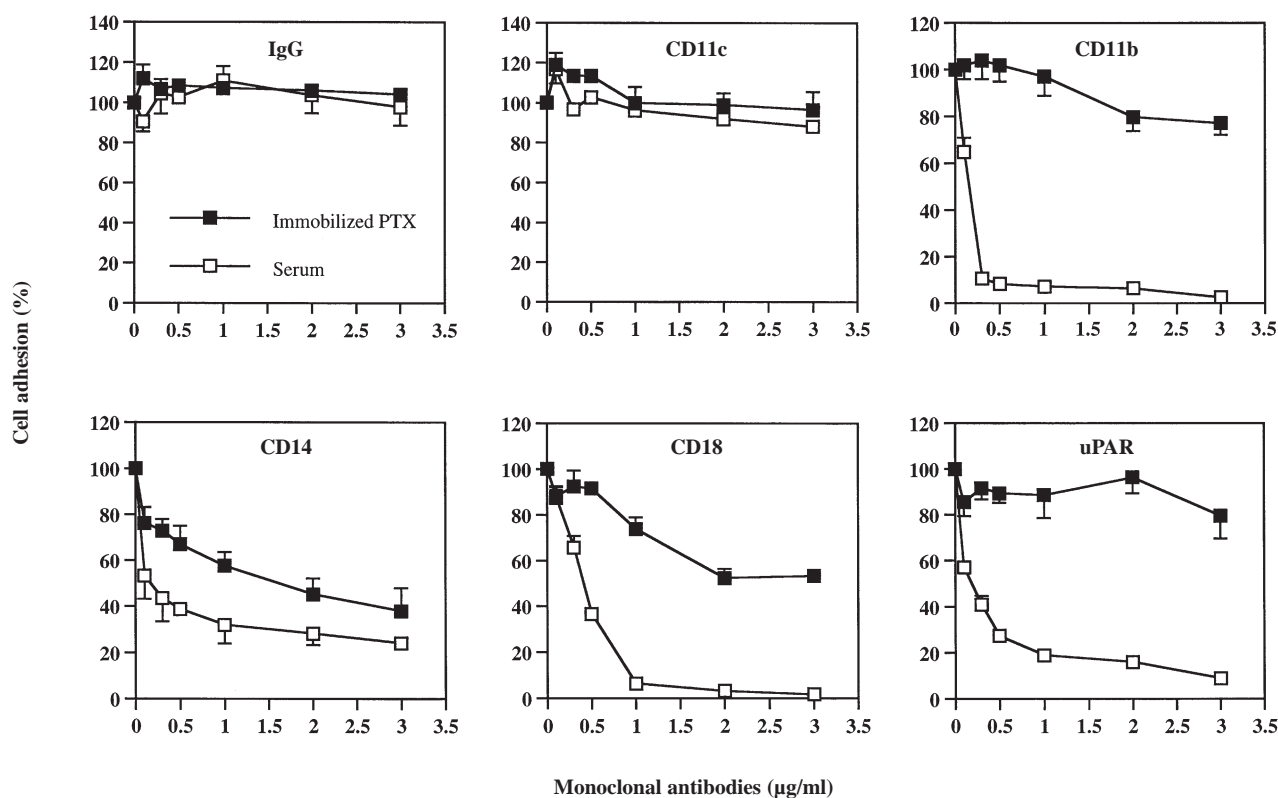


(B)



**Figure 1.** Pertussis toxin (PTX)-induced myeloid cell adhesion in serum. (A) Representative photographs showing PTX-induced myeloid cell adhesion in a 96-well plate. Panel (a) represents unprimed U937 cells and panels (b) to (i) represent transforming growth factor- $\beta_1$ /1,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> (TGF- $\beta_1$ /D<sub>3</sub>)-primed U937 cells. Panels (a) to (h) display the adherent responses of myeloid cells to PTX holotoxin (10  $\mu$ g/ml) in the presence and absence of mouse monoclonal antibodies (mAbs) (2  $\mu$ g/ml) directed against CD11c, CD11b, CD14, CD18 or urokinase receptor (uPAR). Panel (i) shows the adherent response of myeloid cells to PTX B-oligomer (3  $\mu$ g/ml) alone. (B) Cumulative results showing the effects of different mAbs on PTX-induced myeloid cell adhesion. Each value represents the mean  $\pm$  SEM of four to six experiments performed in duplicate and is expressed as the percentage of control adherent responses to PTX. \* $P < 0.05$  compared with primed U937 cell controls. IgG, immunoglobulin G.

Ca<sup>2+</sup> fluorescence intensity in myeloid cells treated with PTX. As shown in Fig. 4(B), PTX induced a substantially greater increase of [Ca<sup>2+</sup>]<sub>i</sub> in the TGF- $\beta_1$ /D<sub>3</sub>-primed U937 cells than in

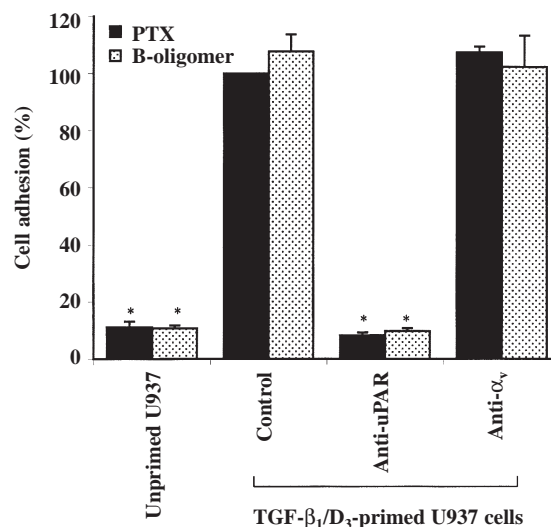


**Figure 2.** Concentration–response studies of the effects of different monoclonal antibodies (mAbs) on pertussis toxin (PTX) (10 µg/ml)-induced primed U937 cell adhesion in serum and on direct cell binding to immobilized PTX (10 µg/ml). Each value represents the mean  $\pm$  SEM of four to six experiments performed in duplicate and is expressed as the percentage of control adherent responses to PTX. IgG, immunoglobulin G; uPAR, urokinase receptor.

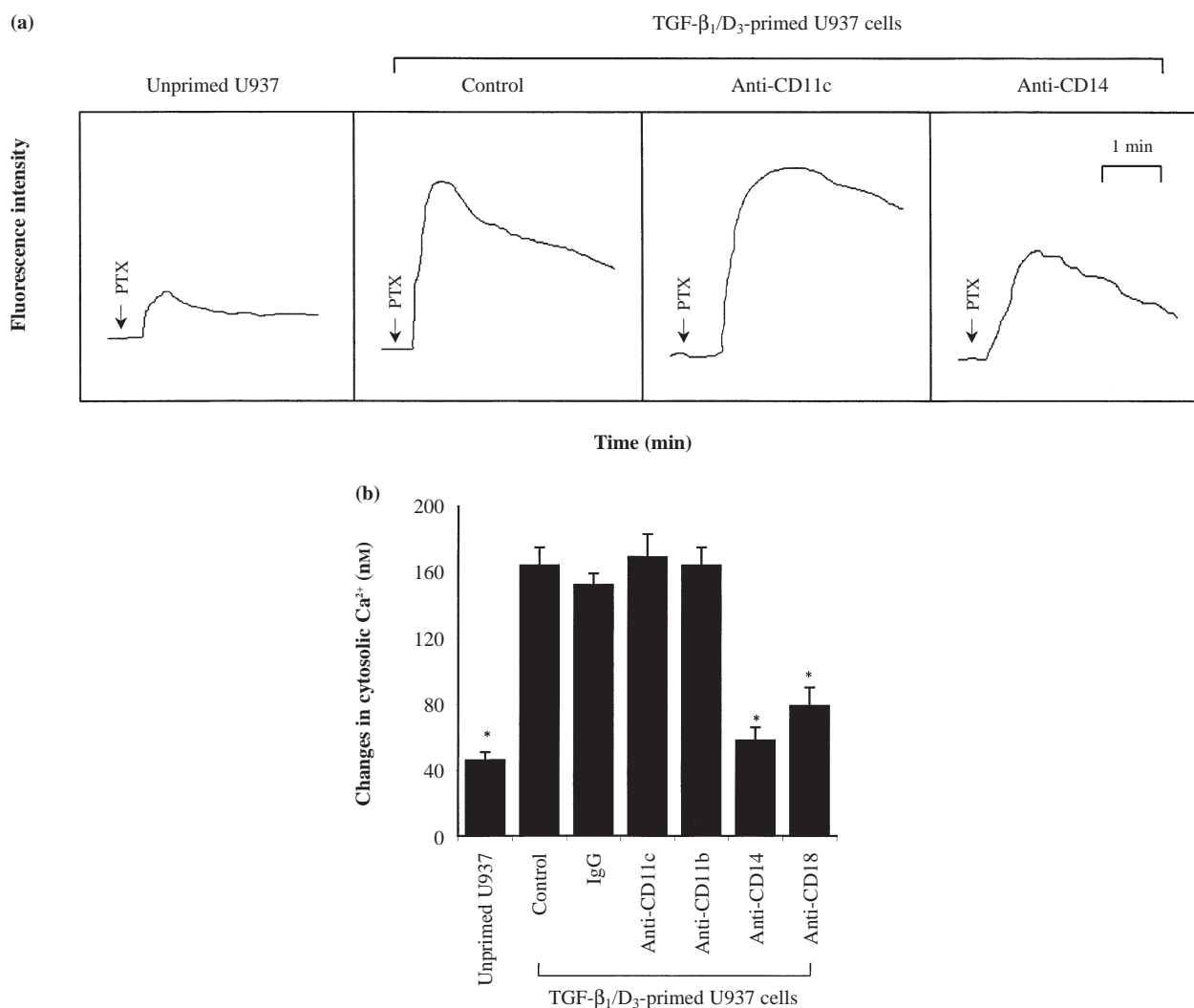
the unprimed U937 cells. The PTX-induced rise of  $[Ca^{2+}]_i$  was significantly inhibited by anti-CD14 mAb and, to a lesser extent, by anti-CD18 mAb. Whereas mAbs against CD11c and CD11b failed to block the increase of  $[Ca^{2+}]_i$  induced by PTX. These observations indicate that CD14, and probably CD18, are the potential binding sites for PTX on myelomonocytic cells.

#### PTX-binding site on myelomonocytic cells

To obtain direct identification of the PTX-binding site on primed U937 cells, we conducted radiolabelled receptor-isolation experiments using biotinylated PTX. Our autoradiography results (Fig. 5) show that the biotinylated PTX isolated a protein of 48 000 MW from the primed U937 cells (lane 2), which is of the same molecular weight as human CD14 antigen isolated from HL-60 cells induced to differentiate to monocytes.<sup>21</sup> This protein band disappeared when excess unlabelled PTX (lane 3) or anti-CD14 (lane 5), but not anti-CD11b (lane 4) or anti-CD18 (lane 6), was added to the receptor precipitation mixture. As expected, biotinylated PTX did not detect the 48 000-MW protein in unprimed U937 cells (lane 1) because the expression of CD14 in control U937 cells was very low. Taken together, our findings show that CD14 is a binding site for PTX on myelomonocytic cells.



**Figure 3.** Pertussis toxin (PTX)-induced primed U937 cell adhesion to vitronectin (5 µg/ml)-coated plates. U937 cells were treated with PTX holotoxin (10 µg/ml) or PTX B-oligomer (3 µg/ml) in the presence or absence of monoclonal antibodies (mAbs) directed against domain II of the urokinase receptor (uPAR) (2 µg/ml) or  $\alpha_v$  integrin (1:100 dilution). Each value represents the mean  $\pm$  SEM of four to six experiments performed in duplicate and is expressed as the percentage of control adherent responses to PTX. \* $P < 0.05$  compared with primed U937 cell controls. D<sub>3</sub>, 1,25-(OH)<sub>2</sub> vitamin D<sub>3</sub>; TGF- $\beta_1$ , transforming growth factor- $\beta_1$ .



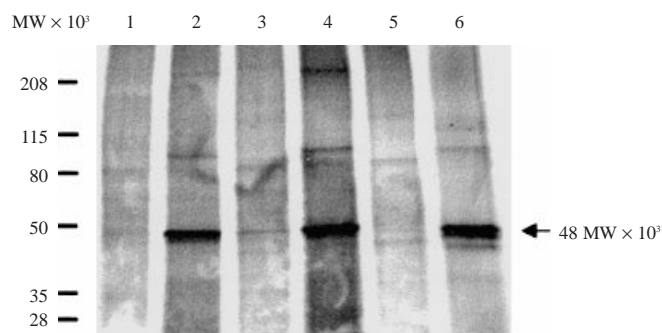
**Figure 4.** Pertussis toxin (PTX)-induced increase of intracellular calcium concentration ( $[Ca^{2+}]_i$ ) in primed U937 cells in the presence and absence of mouse monoclonal antibodies (mAbs) (2  $\mu$ g/ml) directed against CD11c, CD11b, CD14 or CD18. Cells ( $10^6$ /ml) were loaded with the  $[Ca^{2+}]_i$  indicator dye, Fluo-3/AM (10  $\mu$ M), for 1 hr at 37° in RPMI-1640. After incubation with different mAbs for 30 min, aliquots of cell suspension were transferred to cuvettes and then challenged with PTX (10  $\mu$ g/ml). Changes in  $Ca^{2+}$ -specific fluorescence were measured using a spectrofluorometer. (a) Representative tracings of changes in cytosolic  $Ca^{2+}$  in myeloid cells, induced by PTX, as reflected by the changes in fluorescence intensity. (b) Cumulative results showing the changes in cytosolic  $Ca^{2+}$  (nm) in myeloid cells induced by PTX. Basal  $Ca^{2+}$  concentration was  $117 \pm 6$  nm ( $n=7$ ). Each value represents the mean  $\pm$  SEM of four to six experiments performed in duplicate. \* $P < 0.05$  compared with control, unprimed, U937 cells. D<sub>3</sub>, 1,25-(OH)<sub>2</sub> vitamin D<sub>3</sub>; IgG, immunoglobulin G; TGF- $\beta_1$ , transforming growth factor- $\beta_1$ .

## DISCUSSION

The present study was undertaken to identify the binding site(s) and the adhesion molecule(s) responsible for PTX-induced myelomonocytic cell adhesion. We have presented evidence from functional studies and receptor-isolation experiments to show that CD14 is a binding site for PTX on myelomonocytic cells. Our data showed that anti-CD14 mAb inhibited PTX-induced myeloid cell adhesion to serum in a concentration-dependent manner, which correlates strongly ( $r=0.93$ ) with the prevention of binding of primed U937 cells to PTX-coated plates. In contrast to anti-CD14, mAbs directed against CD11b, CD18 and uPAR dramatically blocked PTX-induced myeloid cell adhesion in serum at a concentration that failed to prevent any substantial binding of primed U937 cells to

immobilized PTX. In our receptor-isolation study, biotinylated PTX was able to cross-link a 48 000-MW protein in TGF- $\beta_1$ /D<sub>3</sub>-primed U937 cells, and the PTX binding to this protein was specifically blocked by a fourfold excess of non-biotinylated PTX or by anti-CD14 mAb. The mouse anti-CD14 mAb used in this study (clone TÜK4) has been characterized by a number of laboratories, confirming its reactivity with the CD14 antigen.<sup>22</sup> This 48 000-MW protein will probably be the same human CD14 antigen lacking the glycosyl-phosphatidylinositol (GPI) linkage isolated from HL-60 cells induced to differentiate to monocytes.<sup>21</sup> In line with the findings of van't Wout *et al.*,<sup>7</sup> that PTX B-oligomer contains a carbohydrate-recognition domain which recognizes Le<sup>a</sup> and Le<sup>x</sup> determinants on human macrophages, CD14 has been identified to possess sialylated carbohydrate moieties at the *N*-glycosylation sites.<sup>23</sup> PTX





**Figure 5.** Isolation of pertussis toxin (PTX) receptor on myelomonocytic cells. U937 cells ( $10^6/\text{ml}$ ) were first primed with transforming growth factor- $\beta_1$ /1,25-(OH) $_2$  vitamin D $_3$  (TGF- $\beta_1$ /D $_3$ ) in RPMI-1640 for 24 hr and then in methionine-free RPMI-1640, together with 100  $\mu\text{Ci}/\text{ml}$  of [ $^{35}\text{S}$ ]methionine, for a further 24 hr. The radiolabelled cells were lysed, precleared with streptavidin-agarose and then incubated with biotinylated PTX (10  $\mu\text{g}/10^6$  cells) in the absence (lane 1, U937 cells; lane 2, primed U937 cells) and presence of a fourfold excess of unlabelled PTX (lane 3), anti-CD11b (lane 4), anti-CD14 (lane 5) or anti-CD18 (lane 6), for 1 hr at room temperature. The PTX-bound receptors were precipitated by streptavidin-agarose, eluted with 0.1 M glycine-HCl, pH 2.8, and then separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (4–20%). The gel was dried and developed by autoradiography. The arrowhead indicates the protein band recognized by biotinylated PTX.

was shown to bind to human platelet GPIb, inducing rapid platelet aggregation and an increase of  $[\text{Ca}^{2+}]_i$ .<sup>12,24</sup> It has been documented that both human platelet GPIb and CD14 are leucine-rich glycoproteins with a similar leucine motif, which is believed to play a role in ligand binding and adhesion.<sup>25</sup> These findings, together with our receptor-binding results, support the role of CD14 as a binding site for PTX on myelomonocytic cells.

Although CD14 belongs to a family of GPI-anchored cell-surface proteins that lack a traditional transmembrane sequence for signal transduction,<sup>26,27</sup> cumulative evidence has shown that CD14 is physically associated with src-related protein tyrosine kinases (e.g. p53/56<sup>lyn</sup> and p56/p59<sup>hck</sup>), the  $\alpha_i/\alpha$  subunit of the heterotrimeric G proteins and  $\beta_2$ -integrin (CD18) for signal transduction.<sup>28–30</sup> Functional studies have also shown that cross-linking of CD14 by lipopolysaccharide (LPS) induces protein tyrosine phosphorylation, MAPK activation, protein kinase C activity and an increase of  $[\text{Ca}^{2+}]_i$  in monocytes and other cell types.<sup>28,31–33</sup> Our previous studies showed that PTX elicited protein tyrosine phosphorylation and an increase of  $[\text{Ca}^{2+}]_i$  in TGF- $\beta_1$ /D $_3$ -primed U937 cells.<sup>15,34</sup> The present study extended these findings by showing that anti-CD14 and anti-CD18, but not anti-CD11b or anti-uPAR, significantly inhibited a PTX-induced rise of  $[\text{Ca}^{2+}]_i$  in primed U937 cells. These observations further support the notion that CD14 is a binding site for PTX on myelomonocytic cells. The inhibitory effect of anti-CD18 on  $[\text{Ca}^{2+}]_i$  might be the result of steric interference, as CD14 and CD18 have been shown to be closely associated.<sup>30,35</sup>

The inhibitory effects elicited by anti-CD11b, anti-CD18 and anti-uPAR on PTX-induced myelomonocytic cell adhesion in serum probably occur at the focal adhesion sites. This can be explained by the findings that at the same concentration which significantly inhibited PTX-induced myelomonocytic cell adhesion in serum, these mAbs failed to prevent the cells from binding to immobilized PTX. Substantial evidence showed that Mac-1 (CD11b/CD18) and uPAR were isolated as a receptor complex from human monocytes<sup>36</sup> and acted as a functional unit to complement each other's activities.<sup>36–38</sup> In addition, uPAR has recently been identified as a high-affinity adhesion molecule for vitronectin,<sup>16</sup> a major extracellular matrix protein

for cell attachment in serum.<sup>19</sup> Our results show that PTX promoted myeloid cell adhesion to vitronectin-coated plates and that the adhesion was abolished by anti-uPAR mAb directed against domain II of the receptor that recognizes vitronectin.<sup>16</sup> We believe that PTX-induced myelomonocytic cell adhesion in serum is specifically mediated by uPAR because the anti- $\alpha_v$  mAb directed against the  $\alpha_v$  chain of other vitronectin receptors (e.g.  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$ )<sup>20</sup> failed to block the adhesion to vitronectin. In addition, the uPAR-mediated adherent response is closely regulated by Mac-1 (CD11b/CD18) as mAbs against CD11b and CD18 also inhibited the PTX-induced myeloid cell adhesion in serum.

Hence, we propose that CD14 is a binding site for PTX on myelomonocytic cells, and uPAR is the adhesion molecule responsible for the PTX-induced myeloid cell adhesion in serum. The regulation of uPAR activity induced by CD14 interaction with PTX may present an early pathological step utilized by *B. pertussis* to down-regulate macrophage migration capacity in the airway and to facilitate bacterial internalization into the macrophages.<sup>39</sup> Further study to investigate the *in vivo* protective effects of anti-CD14 mAb in PTX-induced respiratory tract infection is warranted. In addition, the present study may provide insight into a potential PTX-mediated regulation of HIV infection in myeloid cells, as has been illustrated in T lymphocytes.<sup>13,14</sup>

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