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Pentoxifylline induces the shedding of L-selectin on polymorphonuclear cells by stimulation via adenosine receptor as well as by the inhibition of phosphodiesterase

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Abstract We investigated the effects of pentoxifylline (PTX) on the expression of L-selectin on polymorphonuclear leukocytes (PMN). PTX induced the down-regulation of L-selectin expression in dose- and time-dependent manner. The measurement of soluble L-selectin in the culture medium by ELISA indicated that the down-regulation of L-selectin expression by PTX was due to the shedding of L-selectin from PMN. The mechanism by which PTX induced the shedding of L-selectin was investigated. The concentration of intracellular cyclic AMP (cAMP) was increased after treatment of PMN with PTX. However, an elevation of cAMP induced by dibutyryl cAMP (dbcAMP) as well as other methylxanthine derivatives (caffeine, aminophylline, and theophylline) did not induce the shedding of L-selectin. Although stimulation of the adenosine receptor with 5′-N-ethylcarboxamido-adenosine (NECA) or 5′-(N-cyclopropyl)-carboxamido-adenosine (CPCA) adenosine receptor agonists did not induce the shedding of L-selectin, shedding of L-selectin was demonstrated when PMN was incubated simultaneously with rolipram, a phosphodiesterase (PDE) inhibitor, and CPCA. Moreover, shedding of L-selectin induced by PTX was attenuated by aminophylline, an adenosine receptor antagonist. These results indicated that PTX induces the shedding of L-selectin on PMN by stimulation via the adenosine receptor as well as inhibition of PDE.

Key words cAMP · Cell adhesion molecule · Methylxanthine · Neutrophil · Shedding

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Introduction

Polymorphonuclear leukocytes (PMN) provide a primary line of defense against microbial infection, but they are also implicated in the inflammatory reaction.[1] The migration of PMN into tissue is potentially harmful and contributes to the pathology of inflammation. These processes are initiated by the adherence of circulating PMN to activated vascular endothelial cells mediated by cell adhesion molecules (CAM) such as L-selectin (CD62L) and integrins including CD11/CD18 (β2) on PMN.[2] L-selectin constitutively expresses on PMN and mediates PMN rolling and tethering on vascular endothelium at sites of inflammation. Subsequently, firm adhesion of PMN to the vessel wall occurs via the interaction of the CD11/CD18 integrins to an endothelial ligand such as ICAM-1.[3]

Activation of PMN caused by bacterial components such as bacterial lipopolysaccharides (LPS) and N-formyl-methionyl-leucyl-phenylalanine (FMLP), as well as cytokine or chemokine stimuli such as TNFα, results in the shedding of L-selectin and up-regulation of CD11b and CD18 expression.[4] However, the shedding of L-selectin is also induced by anti-inflammatory drugs, but without the up-regulation of CD11b and CD18 expression.[5] The shedding of L-selectin by anti-inflammatory drugs prevents PMN from rolling on endothelial cells, resulting in anti-inflammatory effects. However, the mechanisms that induce the shedding of L-selectin by anti-inflammatory drugs have not been clearly demonstrated.

Pentoxifylline (PTX) is a methylxanthine derivative used for the treatment of vascular diseases such as claudication or cerebral thrombosis. PTX has an inhibitory effect on phosphodiesterase (PDE). Since PDE hydrolyses cyclic AMP (cAMP) into 5′-adenosine monophosphate, the inhibition of PDE increases intracellular cAMP, which may have various effects such as the relaxation of smooth muscle.[6] Moreover, PTX increases the filterability of blood cells because of a decrease in polymerized actin in leukocytes. In addition to these effects, PTX has anti-inflammatory effects on leukocytes. PTX inhibits neutro-

phil adhesion, degranulation priming and superoxide production. These pharmacological effects of PTX are shared by adenosine receptor agonists.[7] Adenosine receptors A1 and A2 subtypes have been shown to be expressed on PMN.[8,9] Although 2-chloroadenosine (adenosine analogue) has no effect on the shedding of L-selectin, stimulation via the adenosine receptor affects the interaction between the cytoskeleton of PMN and L-selectin molecules, resulting in the inhibition of adherence of PMN with endothelial cells.[10] However, the involvement of an adenosine receptor in the effects of PTX on the expression of CAM on PMN is not fully understood. Although PTX inhibits the up-regulation of CD11b and CD18 expression induced by platelet activating factor (PAF) or FMLP,[11,12] the role of PTX on the expression of L-selectin on PMN has not been demonstrated. In this study, we showed that PTX induced shedding of L-selectin on PMN by stimulation of the adenosine A2 receptor as well as inhibition of PDE.

Materials and methods

Reagents

LPS (*E. coli* 0111:B4), FMLP, caffeine, aminophylline, PTX, 8-phenyltheophylline, dibutyryl cyclic AMP (dbcAMP), rolipram, and 5′-N-ethylcarboxamidoadenosine (NECA) were purchased from Sigma Chemical (St. Louis, MO, USA). 5′-(N-cyclopropyl)-carboxamido-adenosine (CPCA) was purchased from Research Biochemicals International (Natick, MA, USA). TNFα was purchased from Genzyme (Cambridge, MA, USA). Theophylline was purchased from Wako Pure Chemical (Osaka, Japan).

Monoclonal antibodies (mAb)

Antihuman L-selectin mAb was purchased from Chemicon International (Temecula, CA, USA). Antihuman CD11a mAb (25.3, mouse IgG1), antihuman CD11b mAb (Bear 1, IgG1), and antihuman CD18 mAb (7E4, mouse IgG1) were purchased from Immunotech (Marseille, France).

Preparation of PMN

PMN were isolated from heparinized venous blood from nine healthy consenting volunteers with Mono Poly-Resolving Medium (Flow Laboratories McLean, VA, USA) exactly as described by the manufacturer. After isolation, the PMN were washed with phosphate buffered saline (PBS) and resuspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and kept at 4°C until use.

Flow cytometry

Cells (1×10^6 cells/100 µl) suspended in RPMI 1640 medium containing 10% FBS were treated with methylxanthine derivatives or several stimulants and incubated in a CO_2 incubator. In some experiments, PMN treated with methylxanthine derivatives and/or adenosine receptor antagonists were used. Cells were washed twice with ice-cold RPMI 1640 medium containing 5% FBS (washing solution) and maintained at ice-bath temperature during the subsequent preparation of the cells for flow cytometry. The cells were stained for 30 min with a saturating concentration of primary mAb diluted with washing solution, followed by reaction with phycoerythrin-conjugated goat antimouse IgG (Immunotech, Marseille, France) for 30 min. After the reaction, the cells were washed with washing solution and PBS containing 5% FBS, then fixed in ice-cold 1% paraformaldehyde in PBS. The cells were analyzed by flow cytometry using FACStar (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Ten thousand cells/group were analyzed. Data were expressed as follows:

$$\text{Per cent expression of CAM} = \frac{\text{Mean fluorescence intensity (MFI) obtained from treated PMN}}{\text{MFI obtained from untreated PMN}} \times 100$$

Quantification of soluble L-selectin (sL-selectin) and intracellular cAMP

PMN (1×10^6 cells/100 µl) suspended in RPMI 1640 medium containing 10% FBS were treated with PTX for 60 min in a CO_2 incubator, after which the supernatant was collected. The level of sL-selectin in the supernatant was determined using the sL-selectin ELISA kit (Bender Med System, Vienna, Austria). In addition, the concentration of intracellular cAMP was measured by the cAMP enzyme immunoassay system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). These ELISA procedures were carried out according to the manufacturer's instructions. The sensitivity of these assays is 0.3 ng/ml for sL-selectin and 38.4 pg/ml for cAMP.

Statistical analysis

All results were given as mean ± standard deviation (SD) of data from three or four independent experiments. Statistical analyses were carried out using Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

Results

Effects of PTX on the expression of CAM on PMN

The effect of PTX on CAM expression on PMN was examined. Flow cytometric analysis demonstrated that the expression of L-selectin decreased in a concentration-dependent manner after the incubation of PMN with PTX. The expression of L-selectin on PMN decreased signifi-

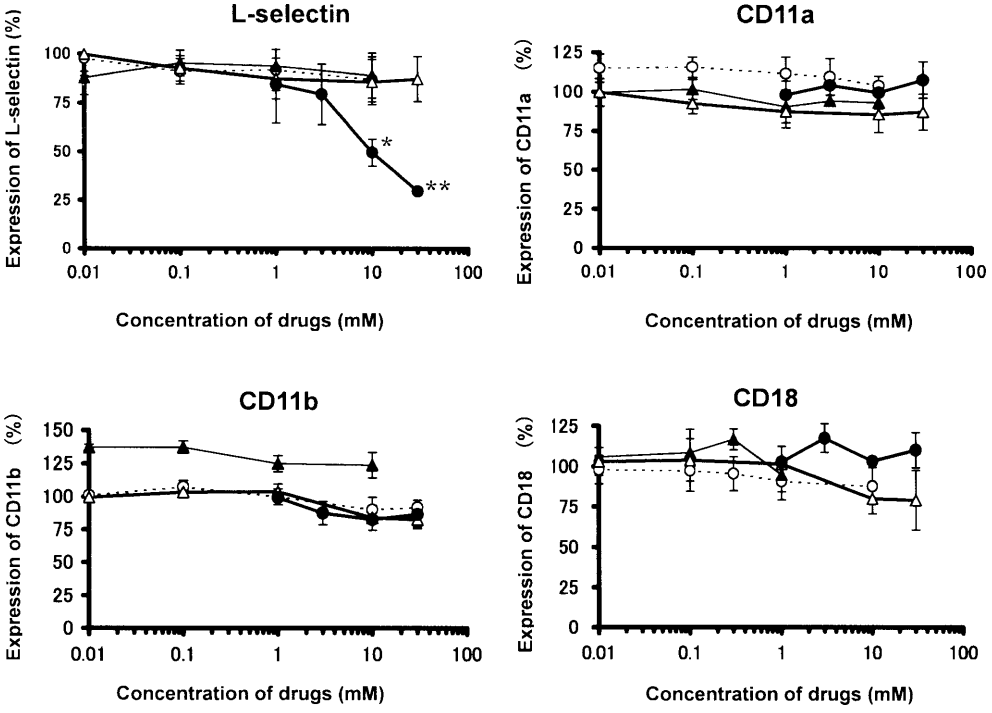

Fig. 1. Effects of methylxanthine derivatives on the expression of cell adhesion molecules (CAM) on polymorphonuclear leukocytes (PMN). The expression of CAM on PMN incubated with methylxanthine derivatives for 30 min was determined by flow cytometry as described in the *Materials and methods* section. Results are presented as mean ± standard deviation (SD) of data from nine independent experiments. *Solid circles*, PTX; *open circles*, theophylline; *solid triangles*, caffeine; *open diamonds*, aminophylline. $*P < 0.01$; $**P < 0.001$

cantly after incubation with 30 mM and 100 mM of PTX. On the other hand, the expressions of CD11a, CD11b, and CD18 on PMN were not changed after incubation of PMN with PTX (Fig. 1). Judging from a trypan blue exclusion assay, the viability of cells is greater than 97.6% after treatment of PMN with PTX. Therefore, the down-regulatory effects of PTX on L-selectin expression were not due to cytotoxicity.

Kinetic analysis of the effects of PTX on the down-regulation of L-selectin expression on PMN was performed. The expression of L-selectin decreased gradually after incubation of PMN with PTX, and was not detected 60 min after incubation of PMN with PTX (30 mM). On the other hand, TNFα (100 ng/ml) induced very rapid shedding of L-selectin on PMN, which was observed 5 min after incubation with TNFα (Fig. 2).

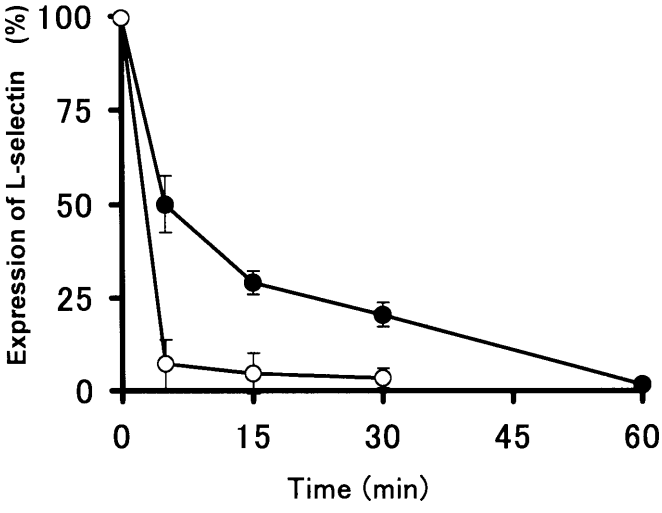

Fig. 2. Kinetic analysis of the expression of L-selectin on PMN. The expression of L-selectin on PMN after incubation with pentoxifylline (PTX) (*solid circles*, 30 mM) or TNFα (*open circles*, 100 ng/ml) was determined by flow cytometry as described in *Materials and methods*. Each value is the mean ± SD of data from three independent experiments

Determination of soluble L-selectin in a supernatant

To examine whether the decreased expression of L-selectin was due to the shedding of L-selectin, the concentration of soluble L-selectin (sL-selectin) in a cell-free supernatant of PMN was determined by ELISA. The level of sL-selectin in the supernatant increased in a concentration-dependent manner with PTX (Table 1). These results indicated that the decreased expression of L-selectin resulted from the shedding of L-selectin on PMN.

Effect of intracellular cyclic AMP on L-selectin expression

PTX is one of the methylxanthine derivatives which have an inhibitory effect on PDE. We therefore examined the effects of caffeine, aminophylline, and theophylline on the expression of L-selectin on PMN to evaluate the involvement of PDE inhibition. However, these methylxanthine derivatives did not affect the L-selectin expression on PMN (Fig. 1).

We determined the content of cAMP in PMN after incubation with PTX or aminophylline to confirm the inhibition of PDE activity in PMN. The concentrations of intracellular cAMP in PMN increased significantly after incubation of

Table 1. Measurement of soluble L-selectin in a culture medium of PMN

		sL-selectin (ng/ml)
PTX	0 mM	18 ± 1.3
PTX	10 mM	38 ± 4.7
PTX	30 mM	56 ± 3.7
PTX	100 mM	78 ± 1.7
TNFα	100 ng/ml	70 ± 1.1

PMN, polymorphonuclear leukocytes; sL-selectin, soluble L-selectin; PTX, pentoxifylline; TNFα, tumor necrosis factor alpha
Each value is the mean ± standard deviation of data from four independent experiments

Table 2. Measurement of cAMP in PMN

		cAMP (pM/10^6 cells)
Unstimulated		0.15 ± 0.01
PTX	30 mM	0.32 ± 0.03*
Aminophylline	30 mM	1.19 ± 0.03**

cAMP, cyclic adenosinemonophosphate; pM, pico molar; PTX, pentoxifylline
Each value is the mean ± standard deviation of data from four independent experiments
* $P < 0.01$; ** $P < 0.001$ compared with unstimulated PMN

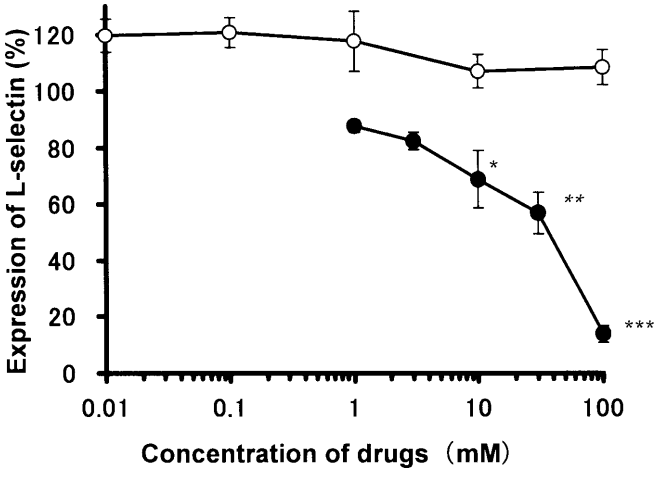

Fig. 3. Effects of dbcAMP on the expression of L-selectin on PMN. The expression of L-selectin on PMN incubated with PTX (*solid circles*) or dbcAMP (*open circles*) for 30 min was determined by flow cytometry as described in *Materials and methods*. Each value is the mean ± SD of data from three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

PMN with PTX (30 mM, 0.32 ± 0.03 pM) or aminophylline (30 mM, 1.19 ± 0.03 pM, Table 2).

Next, we examined the effect of dbcAMP on the expression of L-selectin on PMN since dbcAMP enters into the cells, resulting in an increase in intracellular cAMP. The expression of L-selectin on the PMN did not change after the incubation of PMN with dbcAMP (0.01–100 mM, Fig. 3).

Table 3. Effects of the shedding of L-selectin on PMN induced by PTX

	% stimulated/ unstimulated
NECA 0.1 mM	99.5 ± 2.9
CPCA 1 mM	87.3 ± 11.5
Rolipram 10 mM	80.2 ± 9.8
Rolipram 10 mM + CPCA 1 mM	37.8 ± 7.8**
PTX 30 mM	44.6 ± 7.0*
PTX 30 mM + aminophylline 10 mM	68.6 ± 6.2
PTX 30 mM + aminophylline 100 mM	73.3 ± 10.8
8-Phenyltheophylline 30 μM + PTX 30 mM	28.7 ± 0.6**

PMN, polymorphonuclear leukocytes; NECA, 5′-N-ethylcarboxamidoadenosine; PTX, pentoxifylline; CPCA, 5′-(N-cyclopropyl)-carboxamidoadenosine
Each value is the mean ± standard deviation of data from four independent experiments
* $P < 0.01$; ** $P < 0.001$ compared with unstimulated PMN

Effects of adenosine receptor agonists and PDE inhibitor on L-selectin expression

The pharmacological effects of PTX are shared by adenosine receptor agonists. Caffeine, aminophylline, and theophylline have antagonistic effects on adenosine receptors.[13,14] However, it was known that propentofylline (1-[5′-oxohexyl]-3-methyl-7-propylxanthine) has agonist effects on the adenosine A2a receptor.[15] These reports led us to hypothesize that PTX as propentofylline also has agonistic effects on adenosine receptors and induced the shedding of L-selectin on PMN. We then studied the involvement of adenosine receptors on PMN in the shedding of L-selectin by PTX (Fig. 4, Table 3). An evaluation of the effects of NECA, a nonspecific adenosine receptor agonist, on the expression of L-selectin revealed no change. The expression of L-selectin was weakly reduced when PMN were induced with CPCA, an adenosine A2 agonist (Fig. 4B). The expression of L-selectin decreased by approximately 13% with 1 mM of CPCA (Table 3). When PMN were treated with CPCA (1 mM) and rolipram (10 mM), both PDE inhibitors, simultaneously, the expression of L-selectin was significantly decreased (Fig. 4D), although CPCA or rolipram alone did not induced this reaction (Fig. 4B,C)

Effect of adenosine receptor antagonists on L-selectin expression

Aminophylline has not only an inhibitory effect on PDE, but also a nonselective antagonist effect on the adenosine receptor.[13] As shown in Figs. 1 and 4F, aminophylline itself did not affect L-selectin expression. The shedding of L-selectin induced by PTX was attenuated by aminophylline, a nonselective antagonist for adenosine (Fig. 4G, Table 3). However, 8-phenyltheophylline (30 μM), an adenosine A1 antagonist, did not reduce, but significantly increased, the shedding of L-selectin induced by PTX (Fig. 4H, Table 3).

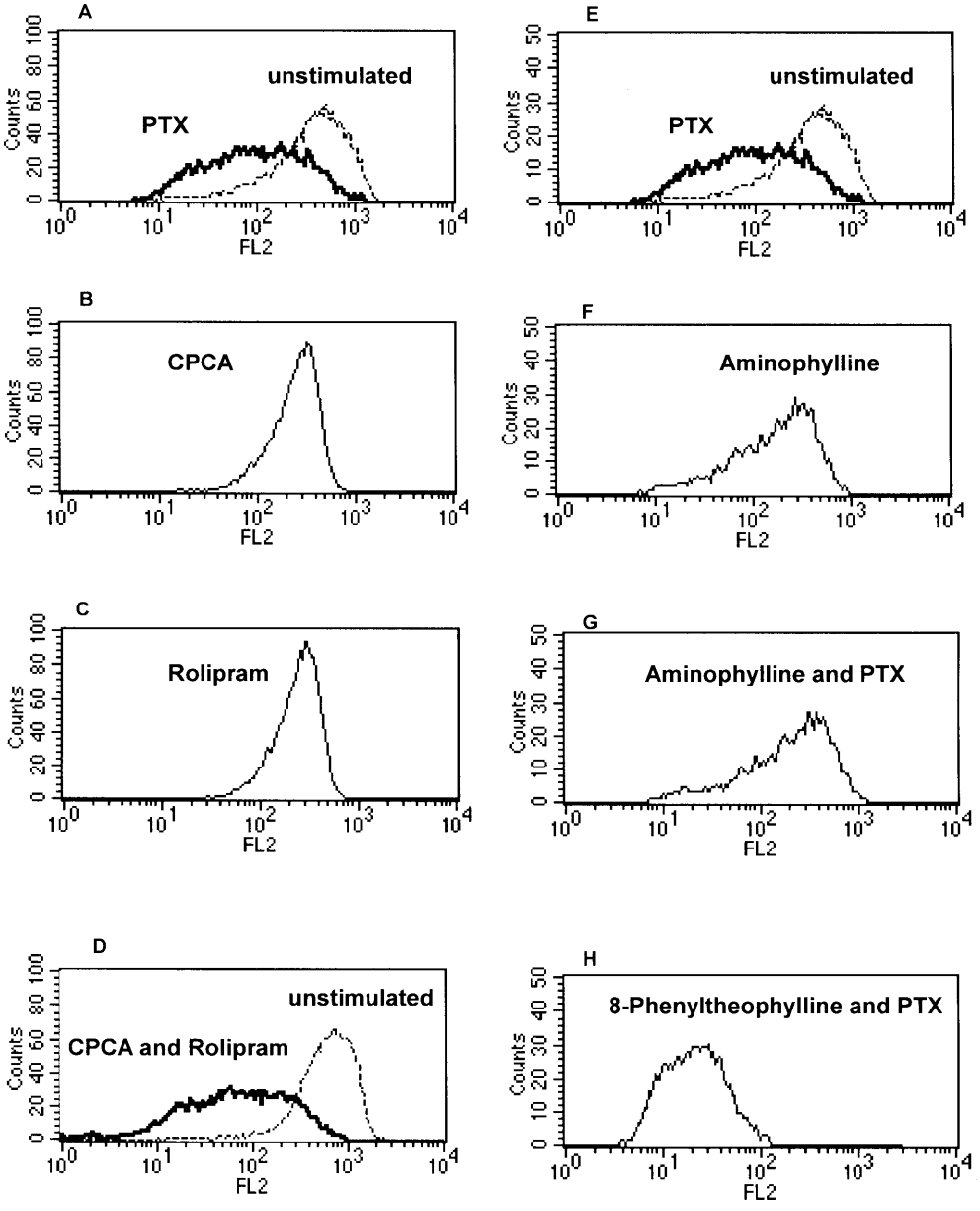

Fig. 4. Effects of L-selectin on PMN by phosphodiesterase (PDE) inhibitor, and adenosine receptor agonists and antagonists. All experiments were incubated with PMN for 30 min, and the expression of L-selectin on PMN was determined by flow cytometory as described in *Materials and methods*. **A** and **E** Unstimulated PMN (*dotted lines*) and treatment with PTX (*solid lines*). **B** Treatment with CPCA (adenosine A2 agonist). **C** Treatment with rolipram (PDE inhibitor). **D** Treatment with both CPCA and rolipram (*solid lines*), and unstimulated PMN (*dotted lines*). **F** Treatment with aminophylline (adenosine nonselective antagonist, PDE inhibitor). **G** Treatment with both aminophylline and PTX. **H** Treatment with both 8-phenyltheophylline (adenosine A1 antagonist) and PTX

Discussion

In this study, we demonstrated that PTX induced the shedding of L-selectin on PMN. The shedding of L-selectin occurs through the activity of anti-inflammatory agents as well as proinflammatory agents such as TNFα, which induced the up-regulation of CD11b and CD18. L-selectin-blocking mAbs significantly reduced the adherence of lymphocytes, neutrophils, monocytes, and eosinophils to cytokine-activated endothelial cells, suggesting that L-selectin is involved in the initial attachment of leukocytes to endothelium. In experiments using the L-selectin-Ig chimera, the blocking of the function of L-selectin inhibited leukocyte rolling in venules of the exterior of the mesentery in acute and chronic inflammation.[16–20] In L-selectin knock-out mice, the number of intracapillary neutrophils was decreased 5 min after the intravenous injection of complement fragments compared with wild-type mice, in which such a decrease was not noted.[21] These reports suggested that the shedding of L-selectin induced by PTX might be one of the anti-inflammatory effects of PTX in vivo.

Among the methylxanthine derivatives examined, only PTX induced the shedding of L-selectin without the up-regulation of CD11b and CD18 expression. The methylxanthine derivatives used in this study inhibited the activity of PDE. On the other hand, caffeine, theophylline, and aminophylline have antagonistic effects on adenosine receptors.[14] Therefore, the adenosine receptor antagonists among the methylxanthine derivatives used in this study did not induce the shedding of L-selectin. The mechanism of the shedding induced by PTX was investigated from two viewpoints: the inhibition of PDE and the stimulation of adenosine receptors. Although the level of cAMP in PMN

treated with PTX was smaller than that in PMN treated with aminophylline (Table 2), aminophylline did not induce the shedding of L-selectin. Incubation of PMN with dbcAMP also did not induce shedding. Therefore, inhibition of PDE or increasing the level of intracellular cAMP itself did not affect the shedding of L-selectin. However, rolipram, a PDE-specific inhibitor, decreased the level of L-selectin expression by approximately 20% (Table 3). On other hand, NECA, a nonspecific adenosine receptor agonist, did not induce the shedding of L-selectin, while CPCA, an adenosine A2 receptor agonist, weakly induced the shedding of L-selectin. Again only an adenosine A2 receptor agonist did not induce the shedding of L-selectin. The shedding of L-selectin was demonstrated only when PMN were incubated simultaneously with rolipram and CPCA (Fig. 4D). These results suggest that PTX induce the shedding of L-selectin by stimulating the adenosine receptor as well as inhibiting the PDE. Aminophylline, a nonspecific adenosine antagonist as well as a PDE inhibitor, attenuated the shedding of L-selectin induced by PTX (Fig. 4G). However, this inhibitory effect was only partial, since even 100 mM of aminophylline did not completely inhibit the shedding (Table 3). These results support the theory that both stimulation of the adenosine receptor and inhibition of PDE are involved in the shedding of L-selectin. PTX and an excess amount of aminophylline increase the intracellular cAMP in PMN. Moreover, stimulation of adenosine A1 receptors decreases the concentration of intracellular cAMP.[9] We speculated that PTX had agonistic action on both adenosine A1 and A2 receptors. Since the binding of PTX with an adenosine A1 receptor is blocked by 8-phenyltheophylline, it was thought that the concentration of intracellular cAMP might be increased, resulting in an enhancement of shedding of L-selectin induced by PTX.

The level of cAMP can also increase by activating adenylate cyclase via stimulation of an adenosine A2 receptor.[14] However, the mere elevation of cAMP could not induce the shedding of L-selectin (Fig. 3). Stimulation via an adenosine A2 receptor also activates the mitogen-activated protein (MAP) kinase.[22] L-selectin may be shed through the activity of ectoenzymes present on the surface of PMN.[4] The activity of these enzymes was inhibited by a hydroxamic acid-based metalloprotease inhibitor.[23] Recently, these ectoenzymes were identified as metalloproteinase-3.[24] It was suggested that the activation of MAP kinase and the elevation of cAMP induced by PTX activated these enzymes, resulting in the shedding of L-selectin. Further analysis of the shedding of L-selectin induced by PTX, and focused on the intracellular signaling cascades, is now being investigated in our laboratory.

In this study, we demonstrated that PTX induced the shedding of L-selectin. Moreover, PTX decreases the production of TNFα by interfering with mRNA accumulation. The inhibitory effects of PTX on TNFα gene transcription are mediated by cAMP.[25] PTX has inhibitory effects on superoxide production from PMN.[11] Although it is controversial whether PTX offers therapeutic benefits for rheumatoid arthritis,[26,27] PTX inhibits experimental allergic encephalomyelitis.[28] PTX exerts a variety of anti-inflammatory actions via both stimulation of adenosine A2 receptors and inhibition of PDE, suggesting that PTX might be a unique therapeutic agent for the treatment of inflammatory diseases.

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