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Contrasting influence of peplomycin and azelastine hydrochloride (Azeptin) on reactive oxygen generation in polymorphonuclear leukocytes, cytokine generation in lymphocytes, and collagen synthesis in fibroblasts

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Abstract The influence of peplomycin (PLM) and azelastine hydrochloride (Azeptin) on reactive oxygen (RO) and cytokine generation was examined in human peripheral blood mononuclear leukocytes, polymorphonuclear leukocytes (PMN), and rabbit alveolar macrophages (RAM). In addition, the influence of these drugs on DNA and collagen synthesis was investigated in human gingival and rabbit pulmonary fibroblasts. In vitro, PLM increased the FMLP- and PMA-induced chemiluminescence and superoxide (O_2^-) generation in human PMN and RAM in a dose-dependent manner. In contrast to PLM, Azeptin dose-dependently suppressed RO generation. Such contrasting actions of PLM and Azeptin were also observed in RAM and PMN obtained from rabbits treated with PLM or Azeptin. Even when human PMN were preincubated with 10–100 μg/ml of PLM, the increase in RO generation was negligible in the presence of 10^{-5} *M* Azeptin in the culture medium. No increases in RO generation were observed in RAM or PMN obtained from rabbits that had received PLM (0.1 mg/kg per day) and Azeptin (0.04 mg/kg per day) concomitantly. PLM suppressed superoxide dismutase activity in RAM and human PMN, while Azeptin did not affect this activity. In vitro, PLM up-regulated the release of interleukin-1 beta, interleukin-6, tumor necrosis factor alpha, and granulocyte-macrophage colony-stimulating factor both from human cells and from RAM and pulmonary fibroblasts. In the generation of these cytokines, Azeptin abrogated the up-regulatory action of PLM. PLM and Azeptin also had contrasting actions in [^{3}H]thymidine and [^{3}H]proline incorporation in human and rabbit fibroblasts. Furthermore, protein tyrosine phosphorylation, in particular that of a 115-kDa protein in human PMN, was suppressed by Azeptin and enhanced by PLM. These results seem to indicate that up-regulated RO and collagen generation are the causative factors of PLM-induced pulmonary fibrosis and that Azeptin may suppress the adverse effect.

Key words Peplomycin · Azelastine hydrochloride
Reactive oxygen

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Introduction

Peplomycin (PLM), a derivative of bleomycin, has commonly been used in the treatment of squamous cell carcinomas, especially for chemotherapy of head and neck carcinomas, in Japan. However, PLM occasionally causes lethal lung fibrosis [9]. Apart from this lethal adverse effect, PLM also induces severe mucositis in the oral cavity and respiratory tract [24]. These adverse actions of PLM are dose-limiting in cancer therapy. The etiological mechanism underlying these adverse actions is not yet sufficiently understood, although enhanced generation of reactive oxygen species (ROS) is suspected to be one of the etiologic factors [6, 7, 19].

Safe use of PLM, which has excellent therapeutic effects on squamous cell carcinoma, depends on prevention of these adverse effects. Of the many agents used clinically to suppress leukocyte function, azelastine hydrochloride (Azeptin), which is used in allergic rhinitis [12], oral ulcers associated with Behçet's disease [25], and asthma [5], is of particular interest, as it is particularly effective in this regard; our previous investigation [28] revealed that Azeptin protected the cell membranes against hypotonic shock and hydrogen peroxide attack. In view of the actions of Azeptin on polymorphonuclear leukocytes (PMN) and alveolar macrophages, we would expect this agent to exhibit actions opposite to those of PLM. Accordingly, in this study, we examined the actions of PLM and Azeptin on ROS and cytokine release from PMN and alveolar macrophages, and we also investigated the influence of both drugs on human and rabbit fibroblasts. The results indicate that Azeptin has superior prophylactic properties against PLM-induced mucositis and pulmonary fibrosis.

Materials and methods

Cell preparation

PMN were isolated from heparinized peripheral blood. After centrifugation on Ficoll-Paque (Pharmacia Fine Chemical, Piscataway, N.J.) gradients, the PMN layer was collected and erythrocytes were sedimented in PBS containing 3% (w/v) dextran. Residual erythrocytes were removed by hypotonic shock. PMN purity of more than 95% and cell viability of more than 98% were microscopically confirmed by Giemsa staining and trypan blue exclusion, respectively. Peripheral blood mononuclear leukocytes (PBL) were collected from the upper layer of the Ficoll-Paque gradients, and were washed three times with PBS. Lymphocytes were separated from monocytes which were allowed to adhere to plastic dish surfaces for 1 h.

Human and rabbit (Japanese white rabbit) fibroblasts were separated from healthy human gingivae and from the lungs of adult rabbits. The resected tissues were minced to pieces of about 2 mm, and these were cultured in DMEM containing 10% FBS. After two to three passages, the proliferated fibroblasts were subjected to the experimental procedures.

Rabbit alveolar macrophages (RAM) were obtained from bronchial lavage fluid by the method of Reynolds et al. [21].

Chemiluminescence

Chemiluminescence was measured with a calcium analyzer (CAF-100, JASCO, Tokyo, Japan). Cells suspended (5×10^5 cells/ml) in HBSS containing 100 μM luminol were incubated for 1 min at 37° C, after which 50 ng/ml of phorbol myristate acetate (PMA; Sigma, St. Louis, Mo.) or 10^{-7} *M* of formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma) was added. Activity was expressed as peak chemiluminescence intensity (mV).

Superoxide (O_2^-) generation

O_2^- was assayed spectrophotometrically by a cytochrome C reduction method. Cells (1×10^7 cells/ml) suspended in HBSS and 100 μ*M* cytochrome C (type VI, Sigma) were poured into each cuvette to give a final cell concentration of 1×10^6 cells/ml. The reaction mixtures in the cuvettes were preincubated at 37° C for 1 min, and 50 ng/ml PMA was then added. The kinetics of cytochrome C reduction were measured by absorbance change at 540–550 nm. The O_2^- concentration was calculated from the linear portion of the cytochrome C reduction curve.

Titration of cytokines

Human lymphocytes, monocytes, fibroblasts, and RAM were cultured in the presence of PLM (5 μg/ml, selected on the basis of plasma levels in patients and our preliminary experiments) or Azeptin (10^{-6} *M*), or both agents, for 48 h. Cytokines in the culture supernatants were measured, using enzyme amplified sensitivity immunoassay kits for interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and granulocyte-macrophage colony-stimulating factor (GM-CSF; Medgenix Diagnostics, Brussels, Belgium) and ELISA kit for interleukin-6 (IL-6; Toray-Fuji Bionics, Tokyo, Japan).

Assay for [^{3}H]thymidine and [^{3}H]proline incorporation

Fibroblasts were cultured in 96-well flat-bottomed microplates. Just before reaching confluence, the cells were treated with PLM and/or Azeptin for 48 h. During the last 4 and 24 h, respectively, 1 μCi/ml [^{3}H]thymidine and 5 μCi/ml [^{3}H]proline with 50 μg/ml ascorbic acid were added. The cells were removed with 0.1% trypsin and harvested on filter paper. The radioactivity of [^{3}H]thymidine or [^{3}H]proline incorporated in the cells was measured with a beta counter.

Following a 24-h pulse, the cells were washed three times with PBS and incubated in 0.1 ml/well of 0.1 *M* NaOH for 5 min at 37° C. The cellular protein in each well was precipitated with 0.1 ml of 10% trichloroacetic acid (TCA) containing 0.4% tannic acid and 5 m*M* L-proline at 0° C for 5 min. Repeating the same procedure three times, 0.2 ml of ethanol-ether (3:1, v/v) mixture was added to each well to remove TCA from the precipitate. The precipitate was redissolved in 0.1 *M* NaOH, and radioactivity in the aliquot was measured with a scintilation counter. The radioactivity was regarded as representing the quantity of synthesized protein. Aliquots were treated with 80 U/ml of type I collagenase (Sigma) for 2 h at 37° C. The radioactivity of the collagenase-soluble fractions was measured and was considered to represent the quantity of synthesized collagen.

Detection of tyrosine phosphorylation

PMN (2×10^6 cells/ml) suspended in HBSS were incubated with various concentrations of PLM and Azeptin at 37° C in the presence or absence of 50 U/ml TNF-α. The reaction was terminated by adding ice-cold 15% TCA solution containing 2 m*M* phenylmethylsulfonyl fluoride and 1 m*M* sodium vanadate. The precipitate was washed with ice-cold ether/ethanol (1/1), dissolved in SDS sample buffer, and subjected to SDS-PAGE. After electrophoresis (30 mA, 3 h), proteins were transferred to an Immobilon-P filter (Millipore), using a Sartorius semi-dry blotting apparatus. After a 60-min incubation in 5% powdered skim milk at room temperature, the filter was incubated with phosphotyrosine-specific monoclonal antibody (40 min). The monoclonal antiphosphotyrosine antibody was detected with peroxidase-conjugated rabbit anti-mouse IgG.

Peroxidase-positive bands were detected using an ECL Western blotting detection system (Amersham). After staining with Coomassie brilliant blue, the molecular weights of proteins were determined using Daiichi-Kagaku standards.

Superoxide dismutase activity

Superoxide dismutase (SOD) activity was measured by the Õyanagi method [17]. The sample (0.1 ml) was mixed with reagent A (0.2 ml: pH 7.0, 0.2 m*M* hydroxylamine plus 0.2 m*M* hypoxanthine, 1.77 m*M* hydroxylamine *O*-sulfonic acid) and 0.1 ml water. The reaction was started by adding reagent B (0.2 ml: 1.25 U/ml xanthine oxidase and 10^{-4} *M* EDTA-2 Na). This mixture was incubated for 30 min at 37° C, and reagent C (2.0 ml: 30 μg/ml sulfanilic acid, 5 μg/ml *N*-1-naphthylethylenediamine, and 16.7% acetic acid) was added. The final mixture was allowed to stand for 20 min at room temperature, and optical absorption was measured at 550 nm. The resected rabbit lungs were homogenized in HBSS (1 g/ml), and assayed for SOD activity. Protein concentration was ascertained by Lowry's method [15].

Results

RO generation

The chemiluminescence of PMN was increased and suppressed, respectively, by treatment with PLM and Azeptin for a 5-min period (Fig. 1). Although 0.1 μg/ml of PLM did not clearly increase the chemiluminescence of PMN, 1 μg/ml and larger doses of PLM did increase the peak intensity (mV) in a dose-dependent manner. The peak intensities for both FMLP- and PMA-induced chemiluminescence were increased to about double their original levels by 100 μg/ml of PLM; however, the chemiluminescence was suppressed when PMN were treated with 50 μg/ml of PLM for a long period (8 h). Azeptin, in contrast, suppressed chemilumi-

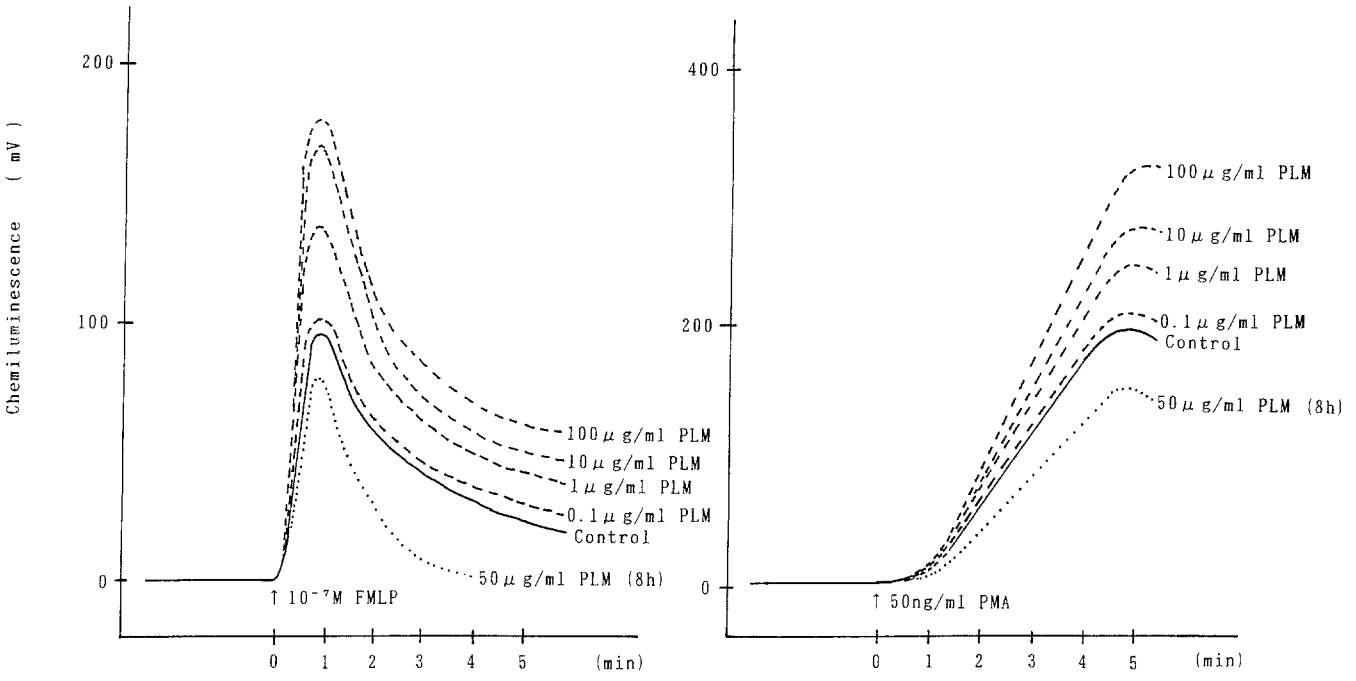

Fig. 1 Influence of in vitro peplomycin (PLM) on formyl-methionyl-leucyl-phenylalanine (FMLP)- and phorbol myristate acetate (PMA)-induced chemiluminescence. Polymorphonuclear leukocytes (PMN) pretreated with the indicated concentrations of PLM for 5 min (– – –) or 8 h (--------) were stimulated with 10^{-7} *M* FMLP or 50 ng/ml PMA

nescence in a dose-dependent manner and abrogated the upregulatory action of PLM (Fig. 2). Even when PMN were preincubated with 100 μg/ml of PLM for 5 min, the peak intensity of FMLP-induced chemiluminescence remained at the control level when 10^{-5} M Azeptin was added to the medium. In PMA-induced chemiluminescence, the up-regulation of peak intensity by 10 μg and 100 μg/ml of PLM was also abrogated by 10^{-5} *M* Azeptin.

The contrasting actions of PLM and Azeptin on reactive oxygen generation were also observed in vivo (Table 1). Compared with alveolar macrophages from untreated (control) rabbits, those from rabbits treated with PLM generated more ROS. For instance, alveolar macrophages from rabbits treated with PLM (0.1 mg/kg per day) showed a peak intensity of 33.0±9.6 mV on FMLP stimulation, while the peak intensity for control macrophages was 22.0±2.0 mV. In contrast, Azeptin significantly suppressed the respiratory burst of alveolar macrophages and peripheral PMN. Control peripheral PMN generated 39.3±4.0 pmol/min of O_2^-, and peripheral PMN from rabbits treated with Azeptin (0.04 mg/kg per day) pro-

Fig. 2A, B Influence of PLM and Azeptin on chemiluminescence in PMN. Peripheral PMN were preincubated with PLM (0–100 μg/ml) and Azeptin (0–10^{-4} *M*) for 5 min, and stimulated with **A** 10^{-7} *M* FMLP or **B** 50 ng/ml PMA. * $P < 0.001$ vs Azeptin 0 (U-test)

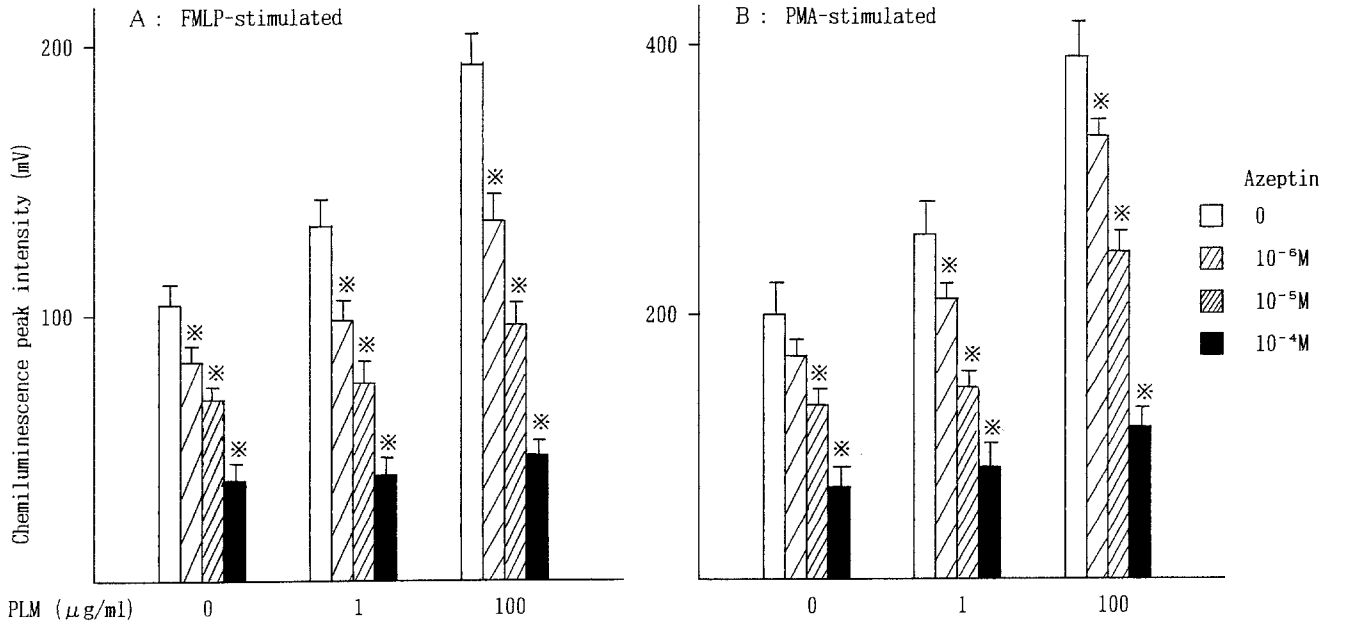

Table 1 Influence of in vivo PLM and Azeptin on reactive oxygen generation (*CL* chemiluminescence; *FMLP* formyl-methionyl-leucyl-phenylalanine; *PMA* phorbol myristate acetate; *PLM* peplomycin)

	Saline (control)	PLM (0.1 mg/kg)	Azeptin (0.04 mg/kg)	PLM + Azeptin (0.1 mg/kg) + (0.04 mg/kg)
Alveolar macrophages				
FMLP-induced CL	22.0±2.0[a]	33.0±9.6*	19.7±0.6*	24.0±5.0
PMA-induced CL	26.0±3.6	29.7±1.5	20.0±1.7*	27.0±2.6
PMA-induced O_2^-	19.0±2.6[b]	20.0±2.6	14.0±5.6*	18.3±2.5
Peripheral PMN				
FMLP-induced CL	45.7±7.1	56.7±10.7*	35.7±6.5*	47.0±15.0
PMA-induced CL	45.0±6.6	49.0±4.4	36.0±4.0*	46.3±10.2
PMA-induced O_2^-	39.3±4.0	45.0±3.6	32.3±2.5*	42.0±3.0

* $P < 0.05$ (vs control, U-test)
[a] Peak intensity (mV)
[b] pmol/min per 10^4 cells

duced 32.3±2.5 pmol/min of O_2^-. When 0.1 mg/kg of PLM and 0.04 mg/kg of Azeptin were injected daily in parallel, the levels of the respiratory burst of alveolar macrophages and peripheral PMN from these rabbits were increased only negligibly, and these levels were almost the same as those in controls.

Cytokine generation

PLM enhanced IL-1β, IL-6, TNF-α and GM-CSF generation of PBL, while, in contrast, Azeptin suppressed generation of these cytokines (Fig. 3). A significant decrease in generation in IL-1β, IL-6, TNF-α, and GM-CSF was produced by 10^{-6} *M* Azeptin, while cytokine generation was significantly up-regulated by 5 μg/ml of PLM. The inhibitory and enhancing effects of Azeptin and PLM, respectively, were also observed in human peripheral monocytes, gingival fibroblasts and RAM. In these cell cultures, the generation of IL-1β, IL-6, and GM-CSF was increased in the presence of 5 μg/ml PLM, and in contrast, was decreased in the presence of 10^{-6} *M* Azeptin, compared with control levels. In addition, in the presence of both 5 μg/ml of PLM and 10^{-6} *M* of Azeptin, less of these cytokines was generated than in controls for all cells examined.

Incorporation of [^{3}H]thymidine and [^{3}H]proline in fibroblasts

PLM (5 μg/ml) and Azeptin (10^{-6} *M*) enhanced and suppressed, respectively, labeled proline and thymidine incorporation (Table 2). In control human gingival fibro-

Fig. 3 Influence of in vitro PLM and Azeptin on cytokine production by human lymphocytes, monocytes, and fibroblasts, and rabbit alveolar macrophages. Cells (1 × 10^6/ml) were cultured with each culture medium in the presence or absence of the reagent indicated for 48 h. *HL* human lymphocytes; *HM* human monocytes; *HF* human fibroblasts; *RAM* rabbit alveolar macrophages. * $P < 0.001$ vs control (U-test)

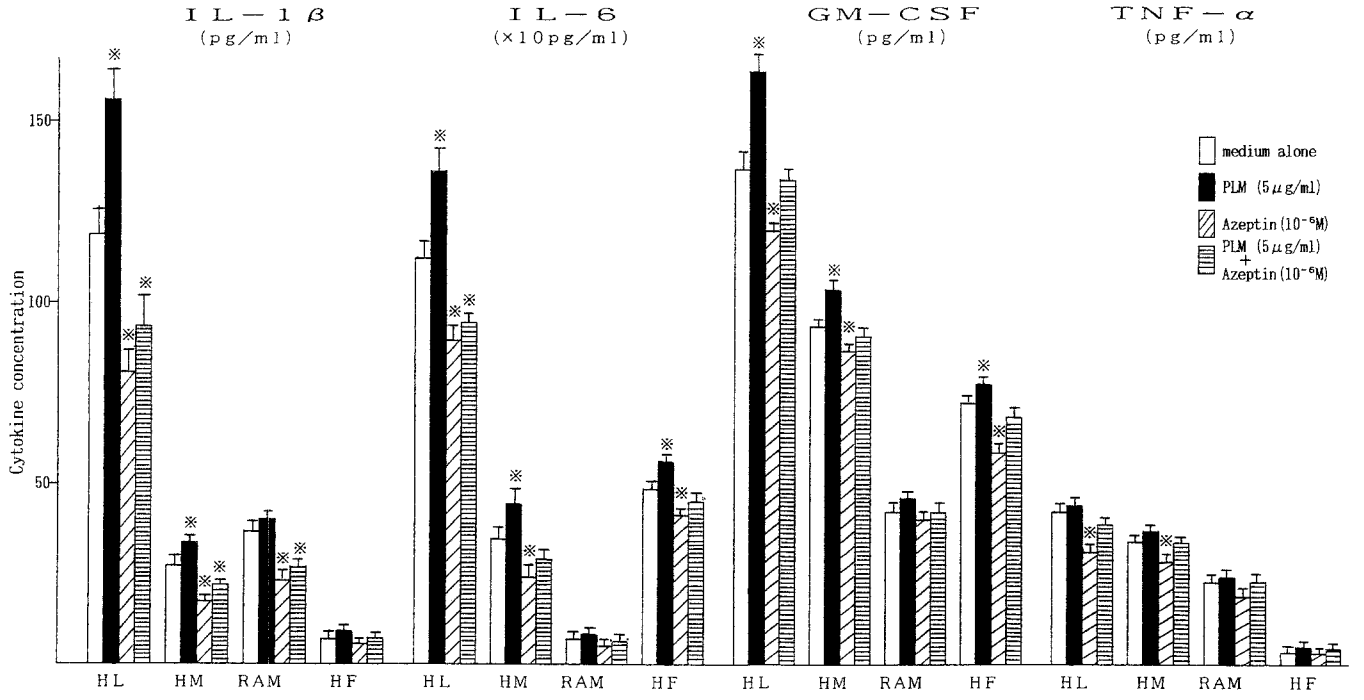

Table 2 Influence of PLM and Azeptin and of the culture supernatants of PLM/Azeptin-treated PBL on collagen and DNA synthesis in fibroblasts (*PBL* peripheral blood lymphocytes)

Fibroblasts	[^{3}H]proline incorporation ($\times 10^3$ dpm/10^4 cells)				[^{3}H]thymidine incorporation ($\times 10^2$ dpm/10^4 cells)			
	Medium	PLM (5 μg/ml)	Azeptin (10^{-6} *M*)	PLM + Azeptin	Medium	PLM (5 μg/ml)	Azeptin (10^{-6} M)	PLM + Azeptin
PLM and Azeptin[a]								
Human gingival	9.7±0.9	11.8±0.8 ↑*	4.1±1.0 ↓*	5.0±0.7	6.6±0.6	8.5±0.6 ↑*	4.9±0.8 ↓*	6.5±0.3
Rabbit pulmonary	10.5±0.8	12.9±0.7 ↑*	8.9±0.6 ↓*	10.3±0.5	——	——	——	——
Supernatants of PLM/Azeptin-treated PBL[b]								
Human gingival	9.5±1.1	12.4±0.7 ↑*	7.2±0.5 ↓*	10.3±1.0	6.3±0.5	7.9±0.4 ↑*	4.9±0.6 ↓*	6.5±0.7
Rabbit pulmonary	10.5±1.1	13.2±0.8 ↑*	7.8±0.7 ↓*	8.6±1.2	——	——	——	——

*$P < 0.05$ (U-test)

[a] Human gingival and rabbit pulmonary fibroblasts separated from four persons and rabbits each were treated with PLM/Azeptin for 48 h, and labeled with [^{3}H]proline and [^{3}H]thymidine during the last 24 h and 6 h of the culture, respectively. The isotope incorporated was then measured. Each examination was performed in triplicated, and the figures in each column are mean ± SD

[b] Human gingival and rabbit pulmonary fibroblasts from four persons and rabbits each were incubated with the culture supernatants of PBL that had been pretreated with PLM/Azeptin. Pulsation of the fibroblasts was the same as above

blasts (without PLM or Azeptin), the value for incorporated thymidine was $6.6 \pm 0.6 \times 10^2$ dpm/10^4 cells, and that for incorporation of [^{3}H]thymidine in gingival fibroblasts pretreated with 5 μg/ml of PLM and 10^{-6} *M* of Azeptin was increased and decreased, to $8.5 \pm 0.6 \times 10^2$ dpm and $4.9 \pm 0.8 \times 10^2$ dpm/10^4 cells, respectively. [^{3}H]Proline incorporation was also affected by PLM and Azeptin in the same manner as [^{3}H]thymidine incorporation. The supernatants of PLM/Azeptin pretreated PBL increased and suppressed protein and DNA synthesis, respectively, in the fibroblasts (Table 2). When human and rabbit fibroblasts were cultured in the presence of the culture supernatants of PBL that had been pretreated with both PLM (5 μg/ml) and Azeptin (10^{-6} *M*), protein and DNA synthesis remained at the control level. Collagen synthesis was correlated with total protein synthesis in all assays.

Protein tyrosine phosphorylation

The tyrosine phosphorylation of a 115-kDa protein and others was increased by PMN treatment with 5–50 μg/ml of PLM for 1 h and suppressed by Azeptin in a dose-dependent manner (Fig. 4). Compared with the bands in lane 1, tyrosine phosphorylation of 108-, 83-, 68-, and 54-kDa proteins in lanes 3 and 5 was increased, but the

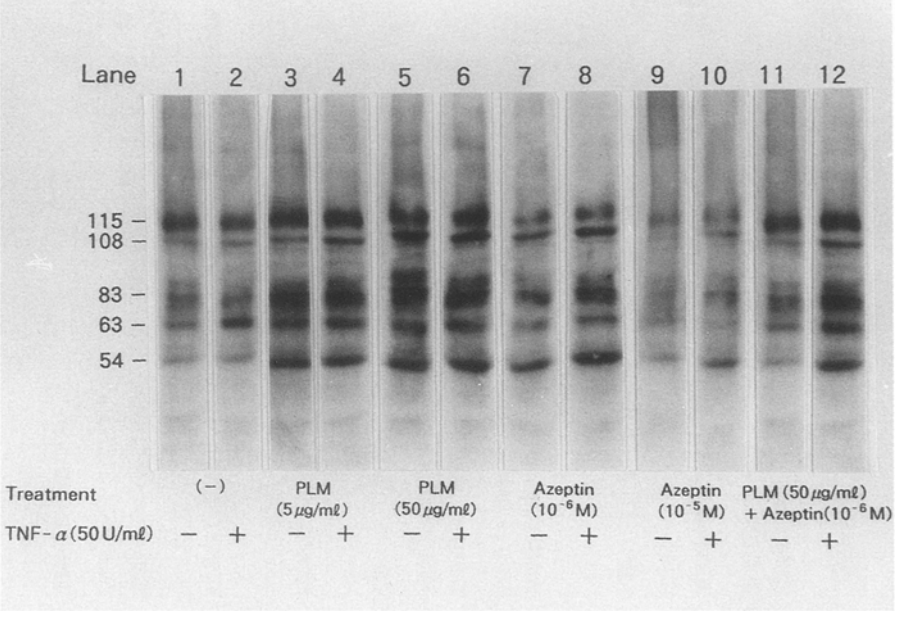

Fig. 4 Tyrosine phosphorylation in PMN treated with PLM and Azeptin. PMN from healthy persons were pretreated with PLM (5–50 μg/ml) and/or Azeptin (10^{-6} or 10^{-5} *M*) in the presence or the absence of 50 U/ml of TNF-α for 1 h. PMN proteins were eluted, electrophoresed and immunoblotted by the method described in "Materials and methods"

Table 3 Influence of in vivo PLM and Azeptin on total superoxide dismutase (SOD) activity. Three rabbits were treated with i.m. injections of saline, 0.1 mg/kg PLM, 0.04 mg/kg Azeptin, or 1.0 mg/kg PLM + 0.04 mg/kg Azeptin every other day (3 injections each in all). Five hours after the third injection, peripheral PMN, alveolar macrophages, and lung tissue specimens were obtained; these were assayed for total SOD activity. Each examination was performed in duplicate, and each value indicates mean ± SD

	Saline (control)	PLM (0.1 mg/kg)	Azeptin (0.04 mg/kg)	PLM + Azeptin (0.1 mg/kg)+(0.04 mg/kg)
RAM	9.60 ± 0.82[a]	7.15 ± 1.20*	9.28 ± 0.82	9.23 ± 0.67
Peripheral PMN	3.97 ± 0.38	3.48 ± 0.46	3.92 ± 0.51	3.83 ± 0.67
Lung tissue	9.02 ± 1.37	7.53 ± 1.16*	8.43 ± 1.69	7.93 ± 1.69

[a] U/mg protein
*$P < 0.05$ (U-test)

115-kDa band was only slightly augmented. In contrast, lane 7 shows a decrease of 115 kDa protein tyrosine phosphorylation, and lane 9 shows largely decreased tyrosine phosphorylation of all proteins indicated. In addition, Azeptin abrogated the up-regulated tyrosine phosphorylation induced by PLM, and densitometry of the 115 kDa band in lane 11 revealed the same fluorescent activity as that in control lane 1 (data not shown).

SOD activity

SOD activity was largely suppressed by PLM injections (Table 3). SOD activity in alveolar macrophages and in the lung tissue from rabbits treated with PLM (0.1 mg/kg per day) by injection was significantly lower than that in untreated controls. However, SOD activity in peripheral PMN remained almost constant, even after PLM injections. Azeptin (0.04 mg/kg) led to a negligible suppression of SOD activity. When both PLM and Azeptin were administered, suppression of SOD activity was very slight.

Discussion

ROS molecules are essential for the protection of the body against microbial invasion [3, 10]. However, these molecules can be hazardous to the tissues, and cells are generally impaired if large amounts of ROS are generated in the tissue and the ROS-scavenging activity of the cells is insufficient. Pulmonary oxygen toxicity is induced by the inhalation of pure oxygen, and some pulmonary diseases associated with ROS have been reported [14, 20, 23, 26]. Investigations have indicated that alveolar epithelial cells are highly susceptible to reactive oxygens [8]. Unfortunately, alveolar macrophages are excellent producers of reactive oxygens and cytokines. Therefore, it is thought that the alveolar tissue is easily impaired when alveolar macrophages and mononuclear cell infiltrates release large amounts of ROS and of cytokines that enhance ROS generation.

Despite its great effectiveness in inhibiting tumor cell growth, the clinical use of PLM is hampered because of its lethal adverse effect, pulmonary fibrosis. Although some basic investigations of this fibrosis have been performed [6, 7, 9, 18, 19, 27], the detailed pathogenesis still remains to be explored.

The present study revealed that PLM enhanced chemiluminescence and O_2^- generation in PMN in a dose-dependent manner and, conversely, suppressed SOD activity, but PLM suppressed RO generation at a high dose (50 μg/ml) and with long-term treatment (8 h). PLM also up-regulated RO generation by RAM. Taking account of the PLM doses used in these experiments and those administered to patients, PLM used clinically seems to actually up-regulate RO generation in the lung.

Protein tyrosine phosphorylation is a crucial event in cell activation, and some kinases and GTP-binding proteins that are essential for RO generation have been reported [11, 16, 22, 30]. The tyrosine phosphorylation of a 115-kDa protein that is considered to play a critical role in RO generation [1] was increased by PLM. As well as being advantageous for RO generation, increased protein tyrosine phosphorylation is also advantageous for cytokine generation. In fact, release of all cytokines examined from lymphocytes was enhanced in the presence of PLM. In the cytokines examined, TNF-α and IL-6 up-regulate RO generation of PMN and monocytes [1, 2, 4, 13, 29]. Therefore, these cytokines appear to up-regulate RO generation indirectly.

Azeptin showed suppressive actions opposite to those of PLM, and Azeptin inhibited the up-regulatory action of PLM in leukocytes. As well as in PMN and PBL, the actions opposite to those of PLM were also observed in the fibroblasts. Protein and DNA synthesis in both pulmonary and gingival fibroblasts were increased at a low concentration (5 μg/ml) of PLM, and the upregulation was significantly inhibited by 10^{-6} *M* Azeptin. The increased collagen synthesis in the fibroblasts seems to be the pathogenic factor in PLM-induced pulmonary fibrosis, and Azeptin seems to protect patients from the lethal fibrosis.

Our results here indicate that impairment of the alveoli by ROS that are generated largely by alveolar macrophages and enhanced collagen synthesis in interstitial fibroblasts account for at least part of the pathogenesis of PLM-induced lung fibrosis. It also appears that Azeptin suppresses the adverse effects of PLM without suppressing the O_2^- scavenger, SOD. To clarify the pathogenesis of PLM-induced pulmonary fibrosis, further in depth exploration of the action of PLM, including its effect on blood vessel

endothelial cells, is needed. The in vivo action of Azeptin in the prophylaxis of this pulmonary fibrosis remained to be clarified.

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