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UPREGULATION OF RESPIRATORY BURST OF POLYMORPHONUCLEAR LEUKOCYTES BY A BLEOMYCIN DERIVATIVE, PEPLOMYCIN

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The influence of peplomycin (PLM) on the respiratory burst of peripheral blood polymorphonuclear leukocytes (PMN) was investigated. Short-term (5 min) treatment of human PMN with 0.1μ g/ml to 100μ g/ml of PLM increased phorbol myristate acetate (PMA)- and formyl-methionyl-leucyl-phenylalanine (FMLP)-induced luminol-dependent chemiluminescence. PMN, as well as alveolar macrophages from rabbits treated with 0.5 to 1.0 mg/kg of peplomycin per day for 5 days, generated more superoxide (O_2^-) than the cells from untreated rabbits. In both PLM-treated and untreated PMN, chemiluminescence induced by FMLP and PMA was decreased to less than 50% of the control by staurosporine, superoxide dismutase (SOD) and catalase. However, the peak intensity in PLM-untreated PMN was decreased to about 30% of the control by genistein, while this agent induced a slight decrease in peak intensity in the PLM-treated PMN. Inositol triphosphate and diacyl glycerol levels were not clearly increased by PLM, but an increase of intracellular Ca^{++} and a shift of protein kinase C (PKC) to the membrane occurred in PMN within 1 min after PLM treatment. Western blotting revealed that the tyrosine phosphorylation of a 115 kDa protein was upregulated by 5 to 50μ g/ml of PLM. While, PLM suppressed SOD activity in alveolar macrophages and PMN. These results seem to indicate that PLM increases the respiratory burst of PMN and macrophages both by way of direct PKC activation and by the upregulation of protein tyrosine phosphorylation. This increased reactive oxygen generation, together with the suppression of SOD activity seems to be tissue-impairing.

KEY WORDS: peplomycin, polymorphonuclear leukocytes, luminol-dependent chemiluminescence, signal transduction, superoxide dismutase

INTRODUCTION

Reactive oxygen species (ROS) play a very important role in the body's defence against microorganisms[1-3]. However, these molecules also impair tissues, and provoke inflammation to a hazardous stage. The negative role of ROS has been demonstrated in many diseases such as nephritis[4], gastrointestinal ulcers[5-7], arthritis[8] and adult respiratory distress syndrome[9,10]. In humans, the brain and pulmonary tissues are those most sensitive to ROS. For example, pulmonary alveoli are very easily damaged by the inhalation of pure O_2 and pulmonary tissue can be impaired by in situ generated ROS[11]. When stimulated appropriately, alveolar macrophages appears to generate high levels of ROS.

Bleomycin (BLM) and its derivative, peplomycin (PLM), are well known to induce lethal pulmonary fibrosis[12-14]. This adverse effect has been explored both clinically and

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basically[15 21], but the actions of these drugs on cytokine and ROS generation still remain to be explored. To clarify the etiology of PLM-induced pulmonary fibrosis, the influence of PLM on ROS release from alveolar macrophages and peripheral blood polymorphonuclear leukocytes (PMN) was examined in the present study. Our findings suggest that PLM-induced pulmonary fibrosis is a result of the increased generation of ROS and cytokines.

MATERIALS AND METHODS

Cell preparations

Human and rabbit PMN were isolated from heparinized peripheral blood according to Böyum's method[22]. After centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) gradients, the PMN pellets were collected, and contaminated erythrocytes were sedimented by the 3% (w/v) dextran. The supernatant fraction was then collected and centrifuged. Any 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. Japanese white rabbit alveolar macrophages were obtained from bronchial lavage fluid by the method of Reynolds *et al.*[23]. The purity and viability were also examined microscopically.

Luminol-dependent chemiluminescence

Chemiluminescence was measured with a calcium analyzer (CAF-100, JASCO Co., Ltd., Tokyo, Japan). Cells suspended in Hanks' balanced salt solution (HBSS) (5×10^5 cells/ml) were cultured for 1 min at 37°C in the presence of 100μ M luminol, and 50 ng/ml phorbol myristate acetate (PMA) (Sigma Chemical, Co., St. Louis, MO) or 10^{-7} M formyl-methionyl-leucyl-phenylalanine (FMLP) (Sigma) was added to the culture medium. The chemiluminescence of the mixture was measured continuously at 37°C. The activity was expressed as the peak intensity (mV) of chemiluminescence.

Superoxide (O_2^-) generation

O_2^- was assayed spectrophotometrically by a cytochrome C reduction method using a Shimadzu UV-300 (Shimadzu Ltd., Kyoto, Japan). Cells (1×10^7 cell/ml) suspended in HBSS and 100μ M cytochrome C (type VI, Sigma) were poured into each cuvette, the final cell concentration being adjusted to 1×10^6 cells/ml. The reaction mixtures in the cuvettes were preincubated at 37°C for 1 min, and 50 ng/ml PMA or 2.5 mg/ml of opsonized zymosan (OZ) was added to the reaction mixtures. The kinetics of cytochrome C reduction were measured by absorbance change at 540–550 nm. O_2^- concentration was calculated from the linear portion of the cytochrome C reduction curve.

Assay of D-myo-inositol 1,4,5-trisphosphate (IP_3), diacyl glycerol (DG) and protein kinase C (PKC)

IP_3, DG and PKC were assayed using Amersham commercial kits (Amersham, U.K.), i.e. a [^{3}H]-labeled IP_3 assay system, DG assay reagent system and PKC enzyme assay system, respectively. Each procedure was followed after the guidance of the kit.

Assay of intracellular Ca^{++} levels

PMN were loaded with 0.1 nmol Fura 2 AM (Dojin Chemical Co., Osaka, Japan) for 30 min at 37°C in the medium (pH7.4) containing 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1 mM $NaHPO_4$, 5.5 mM glucose, and 20 mM HEPES. The cells were then washed twice, and resuspended in HBSS. Intracellular calcium level was measured after stimulation with 10^{-7} M FMLP, using the CAF-100 Ca analyzer.

Detection of tyrosine phosphorylation

PMN suspended in HBSS (2×10^6 cells/ml) were incubated with various concentration of PLM in the presence or absence of 50 U/ml of tumor necrosis factor-alpha (TNF-α) (Genzyme, Cambridge, MA) for 1 h at 37°C. The reaction was terminated by adding ice-cold 15% trichloroacetic acid (TCA) solution containing 2 mM phenylmethylsulfonyl fluoride and 1 mM sodium vanadate. The precipitate was washed with ice-cold ether/ethanol (1/1), dissolved in sodium dodecyl sulfate (SDS) sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis (30 mA, 3 h), the proteins were transferred to an Immobilon-P filter (Millipore) using the Sartorius semidry blotting apparatus. After 60 min incubation in 5% powdered-skim milk at room temperature, the filter was incubated with phosphotyrosine-specific monoclonal antibody (40 min). The monoclonal anti-phosphotyrosine 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, molecular weights of proteins were determined using Daiichi-Kagaku standards.

Superoxide dismutase (SOD) activity

SOD activity was measured by Õyanagi's method[24]. The sample (0.1 ml) was mixed with reagent A (0.2 ml: pH 7.0, 0.2 mM hydroxylamine plus 0.2 mM hypoxanthine, 1.77 mM 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-2Na). The mixture was incubated for 30 min at 37°C and reagent C (2.0 ml: 30μ g/ml sulfanilic acid, 5μ g/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 lungs were homogenized in HBSS (1 g of wet volume/1 ml), and the homogenates were offered to the assay of SOD activity. Total wet volume of resected rabbit lungs were ranged between 220 to 245 g. Protein concentration was determined by Lowry's method[25].

RESULTS

Luminol-dependent chemiluminescence reached a plateau within about 1 and 5 min after stimulation with FMLP and PMA, respectively (Fig. 1). PLM increased the chemiluminescence of PMN in a dose-dependent manner. The peak intensity was increased by short time (5 min) pretreatment of PMN with PLM, however this chemiluminescence was suppressed when PMN were pretreated for a long time (8 h) with a moderately high dose of PLM (50μ g/ml). Without pretreatment, the peak intensity stimulated by FMLP and PMA was less than 100 mV and 200 mV, respectively, while,

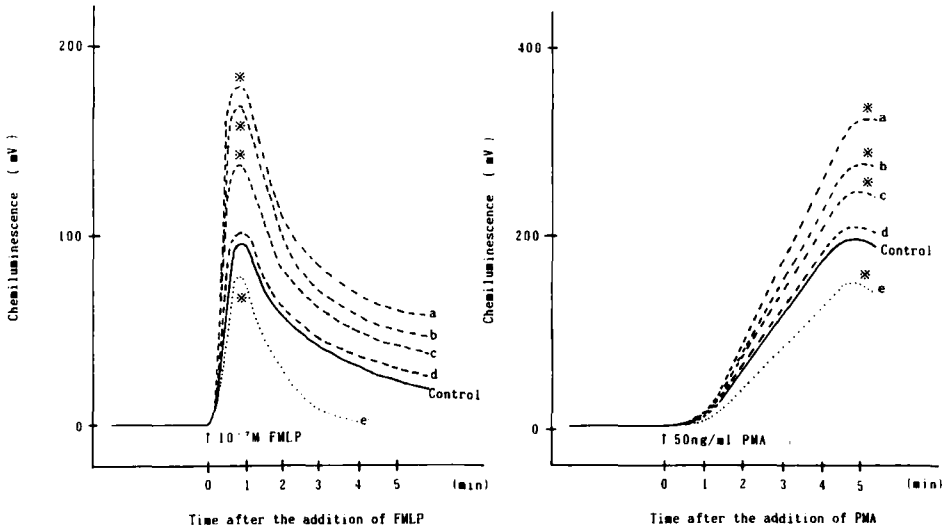

FIGURE. 1. Influence of in vitro PLM on FMLP- and PMA-induced chemiluminescence. Human PMN were pretreated with the indicated doses of PLM for 5 min (– – – –) or 8 h (- - - -), and were then stimulated with 10^{-7} M FMLP or 50 ng/ml PMA. Each data represents the mean of three separate experiments. a ~ d: 5 min treatment (a: 100μ g/ml, b: 10μ g/ml, c: 1μ g/ml, d: 0.1μ g/ml). e: 8 h treatment (50μ g/ml). * $P < 0.05$, vs control, by U-test (at peak intensity).

PMN pretreated with 100μ g/ml PLM for 5 min exhibited a peak intensity of about 180 mV and 320 mV in FMLP and PMA stimulation, respectively.

In vivo PLM upregulated RO generation of PMN and alveolar macrophages (Table 1). Alveolar macrophages obtained from rabbits which received PLM injections (0.1 mg/kg) for 5 days exhibited higher peak intensity than these cells from untreated

TABLE 1.
Influence of in vivo PLM on generation of reactive oxygen species

Cells	Saline (Control)	PLM treatment (0.1mg/kg)	(0.5mg/kg)	(1.0mg/kg)
Alveolar macrophages				
FMLP-induced CL*	22.0 ± 2.0	31.0 ± 9.5	33.4 ± 5.2[a]	35.0 ± 3.0[b]
PMA-induced CL	26.0 ± 3.6	27.7 ± 1.5	31.4 ± 3.2	31.8 ± 2.8
PMA-induced O_2^-†	19.0 ± 2.6	20.0 ± 2.6	23.8 ± 1.7[c]	25.3 ± 2.5[c]
Peripheral PMN				
FMLP-induced CL	45.7 ± 7.1	56.7 ± 10.7	61.2 ± 5.9[c]	62.8 ± 8.4[c]
PMA-induced CL	45.0 ± 6.6	49.0 ± 4 4	59.7 ± 4.8[c]	60.8 ± 8.2[c]
PMA-induced O_2^-	39.3 ± 4.0	44.0 ± 3.6	48.3 ± 4.5[c]	49.5 ± 3.8[c]

Alveolar macrophages and peripheral PMN were isolated from rabbits treated with intramuscular injections of saline or PLM (0.1 mg, 0.5 mg, or 1mg/kg) for 5 days. Luminol-dependent chemiluminescence, stimulated by 10^{-7} M FMLP or 50 ng/ml PMA, was examined. O_2^- generated in the presence of 50 ng/ml PMA was measured by a cytochrome C reduction method. Each examination was duplicated, and each value indicates the mean ± SD of 5 rabbits.

*: CL: Chemiluminescence, Peak intensity (mV)/10^5 cells. a: $P < 0.02$, b: $P < 0.01$, c: $P < 0.05$ (versus control, t-test for all).

†: pmol/min/10^4 cells

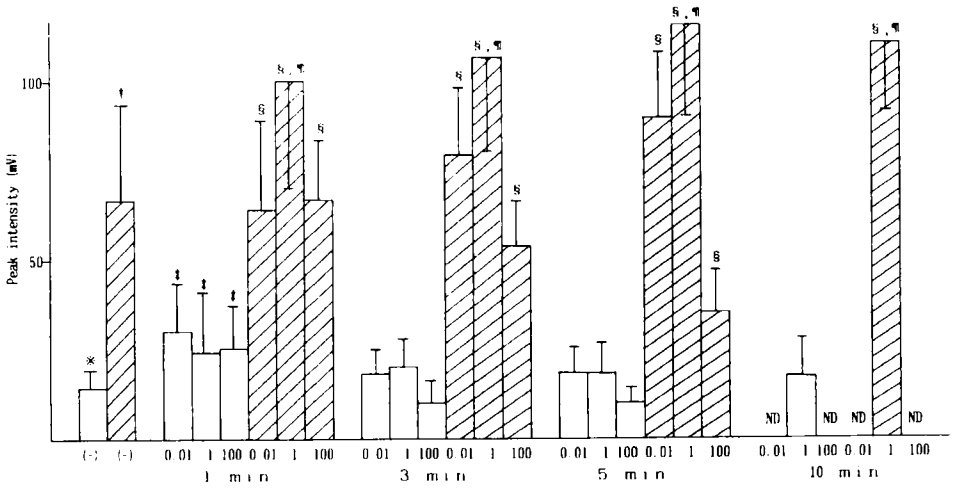

FIGURE. 2. In vivo priming of alveolar macrophages for reactive oxygen generation by PLM. Alveolar macrophages were isolated from 4 PLM-treated (0.25 mg/day× 7, ▨) and untreated (□) rabbits. The cells were cultured in the presence of PLM for the indicated time, and OZ (2.5 mg/ml)-induced chemiluminescence was determined. Each column and bar indicate mean ± SD of duplicate in 4 rabbits. † > *, ‡ > *, § >‡ each corresponding empty column, and ¶ > †. ($P < 0.05$, U-test).

rabbits. When rabbits were treated with 0.5 mg/kg/day or 1 mg/kg/day of PLM, chemiluminescence in the alveolar macrophages was largely increased in response to both FMLP and PMA stimulation. Likewise, PLM induced significant increase in O_2 release from alveolar macrophages. PMN from rabbits treated with PLM also generated a larger amount of ROS than controls from untreated animals.

After injection of PLM (0.25 mg/body/day) for 7 days, rabbit alveolar macrophages were obtained and treated with various doses of PLM for 1, 3, 5, and 10 minutes (Fig. 2). Adding to the in vivo upregulation, a further increase of chemiluminescence was induced in vitro by 1μ g/ml of PLM. However, lower (0.01μ g/ml) and higher (100μ g/ml) doses of PLM did not increase chemiluminescence. This in vivo priming effect of PLM was observed regardless of the in vitro treatment time.

The upregulation of chemiluminescence induced by in vitro PLM was analyzed using enzyme inhibitors and ROS scavengers (Fig. 3). Compared to control (without any agent; 100%), PLM-treated PMN exhibited higher chemiluminescence in both inducers (131% in FMLP and 139% in PMA). When SOD was added to the culture, the peak intensity was decreased to about 38% and 75% of the control intensity induced by FMLP and PMA, respectively. A similar decrease of chemiluminescence was observed with catalase. Methionine (a myeloperoxidase inhibitor), staurosporine (a PKC inhibitor) and 4-bromophenyl bromide (4-BPB, a phospholipase C inhibitor) suppressed chemiluminescence to about 80%, 40 to 60% and 70% of the control level, respectively, in both inducers. There was no difference in suppression by these agents between PLM-untreated and treated cells. However, the degree of suppression induced by genistein was greatly different in PLM-treated and untreated PMN. In the former, the peak intensity was suppressed to less than 30% of the original intensity by the addition of genistein, while in the latter the peak intensity was decreased to 69% of the medium control.

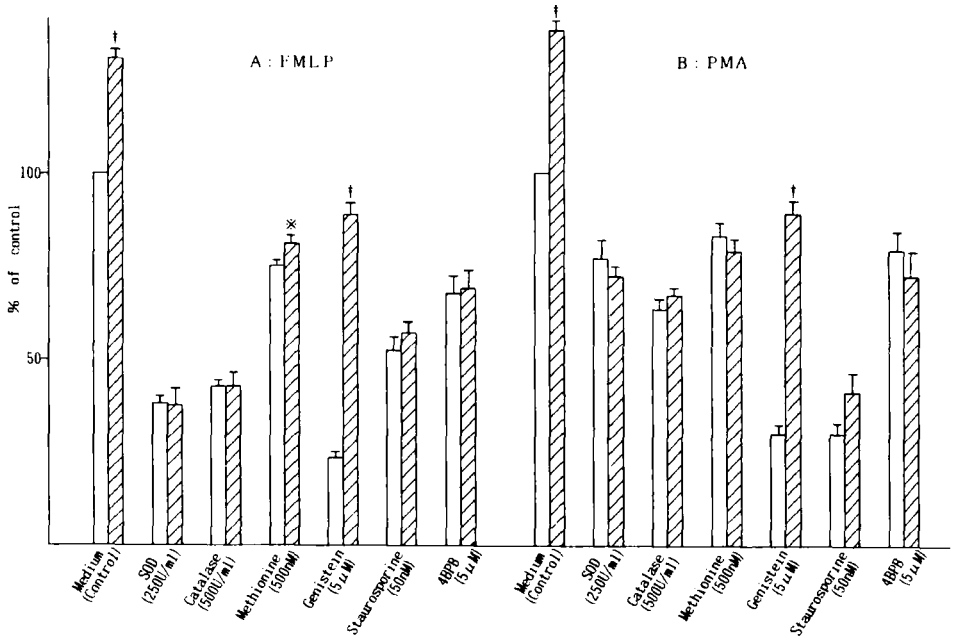

FIGURE. 3. Influence of RO scavengers and enzyme inhibitors on chemiluminescence of PLM-treated PMN. PMN from 4 healthy individuals were pretreated in the presence (⊡) or absence (□) of 5μ g/ml PLM for 1 h, and stimulated with 10^{-7} M FMLP or 50 ng/ml PMA in the presence of the indicated scavenger or enzyme inhibitor. Each column with bar indicates the percentage of

$$\text{control: } \left(\frac{\text{peak intensity in the presence of the agent}}{\text{peak intensity in the absence of the agent}} \times 100 \right) \pm \text{SD}$$

The examination was duplicated. * $P < 0.05$, † $P < 0.001$, vs absence of 5μ g/ml PLM, by U-test.

IP_3 increased rapidly, showing peak level about 15 seconds after FMLP stimulation (Fig. 4), these levels fell down to the original level within 120 seconds after stimulation. PLM slightly influenced IP_3 level. PMN treated with high doses (50 and 100μ g/ml) of PLM exhibited a weak increase in IP_3 levels, while in PMN treated with low doses (0.5 and 5μ g/ml) of PLM IP_3 levels decreased slightly. The influence of PLM on DG activity was different to that on IP_3 level (Fig. 5). The first phase elevation of DG level after the addition of FMLP was inhibited by PMN pretreatment with PLM. The lowering of DG level continued throughout the detection in PMN pretreated with 0.5 and 5μ g/ml PLM. However, in PMN pretreated with 50 and 100μ g/ml PLM, DG level was increased to above the control level 60 sec after the addition of FMLP.

PKC activity in the cytosol of PLM-treated human PMN was lower than that in control PMN (Fig. 6), while conversely, PKC activity in the membrane fraction in PLM-treated cells was increased. These changes in PKC activity occurred within 1 minute after the addition of PLM.

PLM increased $[Ca^{++}]i$ dose-dependently (Fig. 7). $[Ca^{++}]i$ level was increased from 108 nM in the resting condition to 185 nM within one minute of FMLP stimulation. PLM (50μ g/ml)-pretreated PMN had levels of $[Ca^{++}]i$ in excess of 200 nM within a few minutes after the addition of FMLP. This elevated $[Ca^{++}]i$ decreased gradually after being maintained for 10 minutes after stimulation.

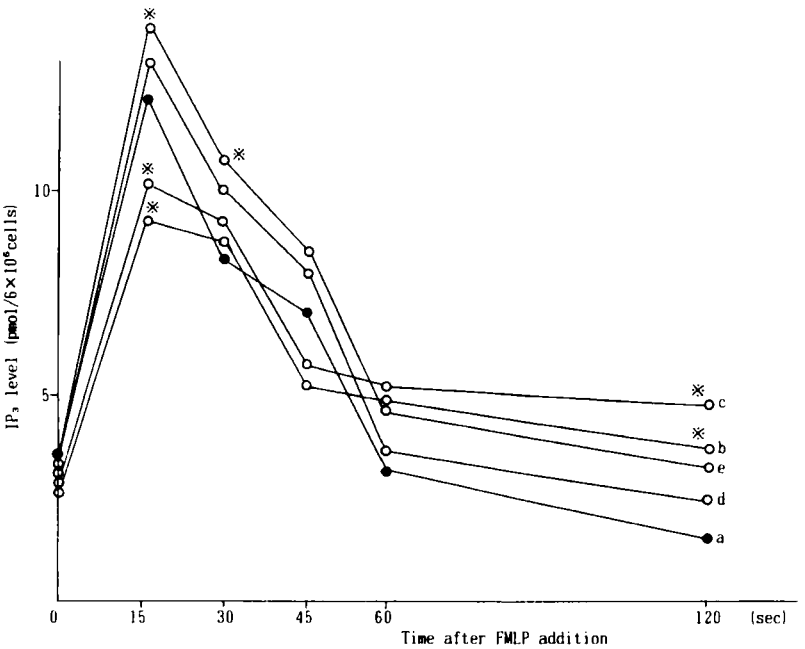

FIGURE. 4. IP_3 levels in PLM-treated human PMN. PMN from 3 healthy individuals were pretreated with the indicated doses of PLM for 1 h, and IP_3 level was determined serially after the addition of FMLP (10^{-7} M). Each examination was duplicated, and each point indicates the mean level of the 3 subjects. a: 0μ g/ml, b: 0.5μ g/ml, c: 5μ g/ml, d: 50μ g/ml, e: 100μ g/ml. * $P < 0.05$, vs control, by U-test.

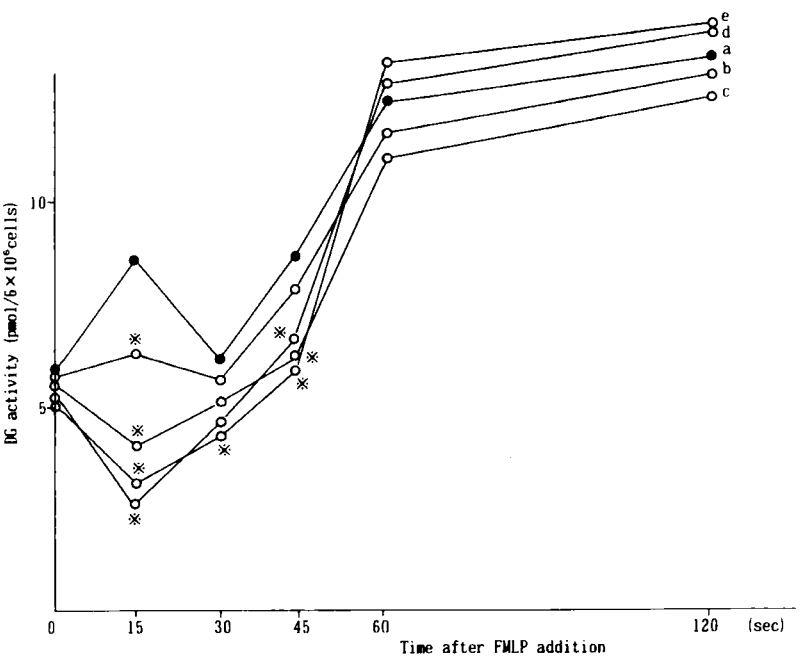

FIGURE. 5. Kinetics of DG levels in PLM-treated human PMN. PMN from 3 healthy individuals were pretreated with the indicated doses of PLM for 1 h, and DG level was determined serially after the addition of FMLP. Each examination was duplicated, and each point indicates the mean level of the 3 donors. a: 0μ g/ml, b: 0.5μ g/ml, c: 5μ g/ml, d: 50μ/g/ml, e: 100μ g/ml. * $P < 0.05$, vs control, by U-test.

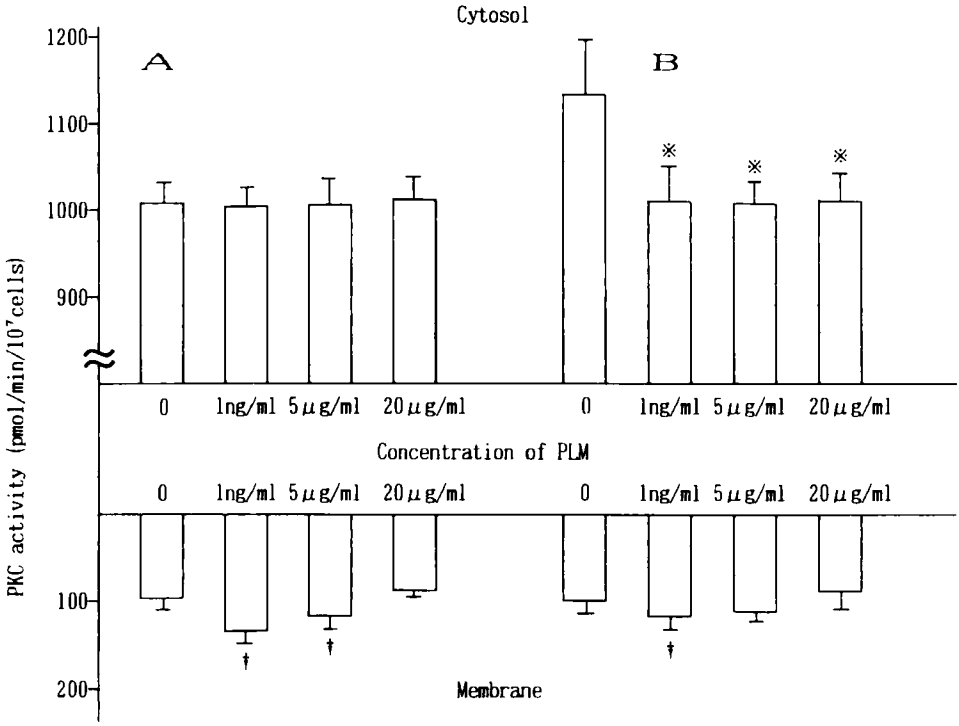

FIGURE. 6. Influence of in vitro PLM on cytosol and membrane PKC activity. PMN from 4 healthy volunteers were incubated with the indicated doses of PLM for 1 (A) or 5 min (B), and bathed in ice cold water. After the cytosol and membrane fractions were separated, PKC activity was assayed with Amersham's Assay Kit. Each column with bar shows the mean ± SD of duplicate experiments for the 4 donors. * and †: significantly lower and higher than the control, respectively ($P < 0.05$, U-test).

Protein tyrosine phosphorylation was amplified by PLM (Fig. 8). When PMN were pretreated with 5 to 100μ g/ml of PLM and stimulated with 50 U/ml TNF-α, the tyrosine phosphorylation of a 115 kDa protein was increased; however, the amplification of the protein phosphorylation by 100μ g/ml of PLM was weakly visible when TNF-α was not added.

SOD activity in alveolar macrophages, PMN, and the lung tissue was not significantly suppressed in samples from animals received intramuscular injections of a low dose of PLM (0.1 mg/kg/day) (Table 2). However, in samples from rabbits received high doses of PLM (0.5 mg/kg/day or 1 mg/kg/day), SOD activity was suppressed. Compared with PMN, alveolar macrophages exhibited more suppressed SOD activity, and the decrease of SOD activity in lung tissue was significant.

DISCUSSION

Although PLM and BLM exhibit excellent antineoplastic action in many epithelial tumors, particularly in squamous cell carcinomas[26 28], both agents cause severe adverse actions, occasionally inducing lethal pulmonary fibrosis[12 14]. This lethal adverse effect obviously limits the use of these drugs. It has been suggested that reactive oxygen

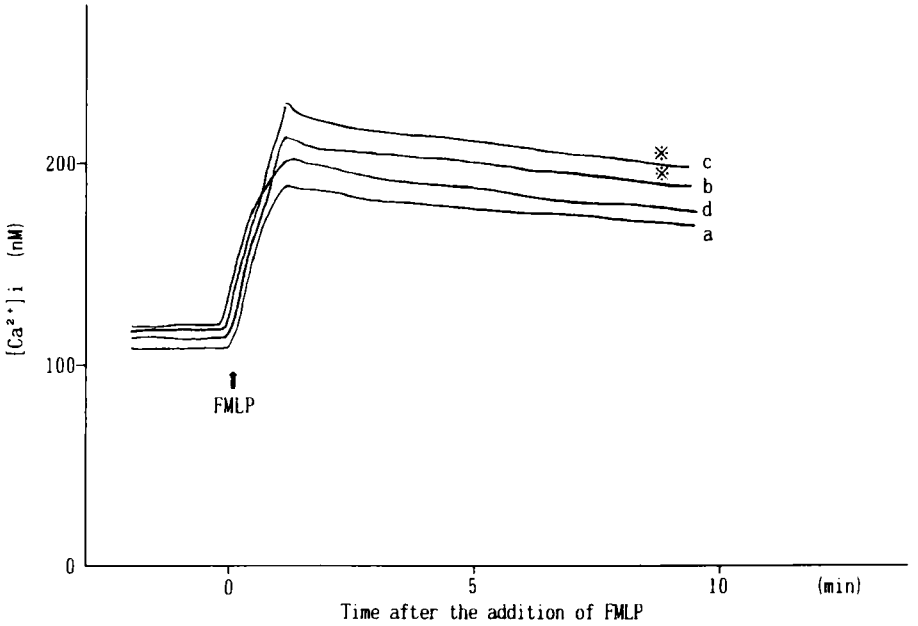

FIGURE. 7. Influence of PLM on $[Ca^{++}]i$ in human PMN. PMN from 2 healthy individuals were pretreated with PLM (0 to 500μ g/ml) for 1 h and loaded with Fura-2 AM during the last 30 min. After stimulation with 10^{-7}M FMLP, $[Ca^{++}]i$ level was measured by a calcium analyzer, CAF-100. Each curve represents the mean of three separate experiments. a: 0μ g/ml, b: 5μ g/ml, c: 50μ g/ml, d: 500μ g/ml. * $P < 0.05$, vs control, by U-test.

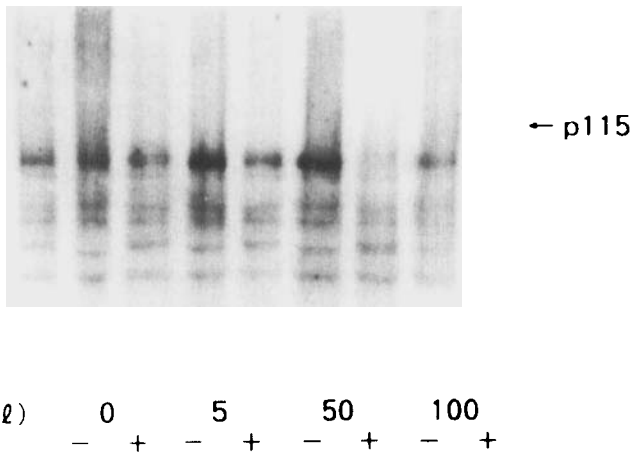

FIGURE. 8. Tyrosine phosphorylation in PMN treated with PLM. PMN from healthy individuals were pretreated with PLM (0 to 100μ g/ml) in the presence or absence of 50 U/ml TNF-α for 1 h at 37°C. The eluted proteins were subjected to SDS-PAGE and transferred to an Immobilon-P filter. Phosphorylated tyrosine residues were detected by immunoblotting with antiphosphotyrosine monoclonal antibody.

TABLE 2.
Influence of in vivo PLM on total SOD activity

Cells and tissue	Saline (Control)	Injection of PLM (0.1mg/kg)	(0.5mg/kg)	(1.0mg/kg)
Alveolar macrophages	4.80 ± 0.82*	3.58 ± 1.20†	3.53 ± 1.12†	3.51 ± 1.34†
Peripheral PMN	3.97 ± 0.38	3.48 ± 0.46	3.35 ± 0.44	3.26 ± 0.51
Lung tissue	9.02 ± 1.37‡	7.83 ± 1.16	7.53 ± 1.49	7.25 ± 1.08†

Three rabbits were treated with intramuscular injections of saline, 0.1mg/kg, 0.5 mg/kg or 1.0 mg/kg PLM every other day by 3 times. Five hours after the third injection, peripheral PMN, alveolar macrophages, and lung tissue specimens were obtained; these were assayed for total SOD activity. Each test was duplicated. Each value indicates the mean ± SD of three rabbits.
*: U/10^7 cells, ‡: U/mg protein, †: $P < 0.05$, (versus control, U-test)

species are the pathogenic agent in pulmonary fibrosis[19,20,29-31] as well as in adult respiratory distress syndrome[9,10]. Indeed, ROS-induced pulmonary alveolar damage is thought to be implicated in PLM-induced pulmonary fibrosis[14]. Accordingly, it appears to be important for the analysis to examine the action of PLM on pulmonary macrophages, leukocytes, endothelial cells and fibroblasts.

Here, it was shown that PLM dose-dependently enhanced the chemiluminescence of PMN in vitro; however, at a high dose (50μ g/ml) and with long term (8 h) treatment, it suppressed chemiluminescence. In vivo, PLM (0.1 to 1 mg/kg) increased ROS release from alveolar macrophages. Apart from this upregulation of ROS generation, PLM exerted a priming effect on OZ-induced chemiluminescence. These results indicate that, when administered clinically, PLM primes and stimulates alveolar macrophages and PMN to produce high levels of ROS.

When PLM-induced ROS were analyzed using ROS-scavengers and enzyme inhibitors, some characteristics of PLM-induced chemiluminescence were clarified. In FMLP, the addition of SOD or catalase to the assay system reduced chemiluminescence to the same degree (about 40% of the control, which indicates that O_2^- and H_2O_2 are main molecules in ROS induced by FMLP) regardless of treatment of PMN with PLM. Similarly, the inhibition of chemiluminescence by methionine, staurosporine, and 4-BPB was not different in PLM-treated and untreated PMN, although staurosporine showed largest suppression of respiratory burst indicating that RO generation largely depends on PKC activity. The effects of genistein differed from those of the other agents. RO generation was not greatly inhibited by genistein and the peak intensity level remained at near 90% of the control when PMN were pretreated with PLM. However, RO generation was suppressed by genistein to about one fourth of the control if PMN was not pretreated with PLM. These results seem to indicate that PLM stimulates PKC and protein tyrosine phosphorylation. In fact, upregulation of tyrosine phosphorylation and PKC activity by PLM was observed in the present study. The PLC inhibitor, 4-BPB, suppressed RO generation to about 70% of the control level. Therefore, PKC seems to be activated by PLM in two different manners, that is, by direct stimulation and via PLC activation.

The inhibition of chemiluminescence by the PKC- and PLC-inhibitors, as well as increase of $[Ca^{++}]i$ and the significant PKC shift from the cytosol to the membrane after short-term PLM treatment of PMN, indicate that PKC activity was upregulated via the second messenger, DG. However, increases of IP_3 and DG were not observed. Therefore, signal transduction via these second messengers was not identified as the main pathway in the stimulation of PLM.

Methionine, a myeloperoxidase inhibitor, more suppressed chemiluminescence in PLM-treated PMN than genistein, being consistent with that contrary to the upregulation of PKC and signal transduction, PLM suppresses myeloperoxidase activity in a dose-dependent manner (data not shown). As shown above, PLM increased chemiluminescence in PMN. Therefore, the upregulation of signal transduction seems to surpass the suppression of myeloperoxidase in PLM-treated PMN.

PLM significantly suppressed SOD activity even at a low dose. Under such condition of suppressed ROS scavenging activity, increased ROS generation is very harmful to tissues. Pulmonary alveoli appear to be easily impaired if high levels of ROS are generated and ROS scavengers are suppressed by PLM because pulmonary alveoli are sensitive to oxygen[32].

Alveolar macrophages are the primary producers of ROS in the lung. Besides, many other ROS-producing cells infiltrate lung tissue. In patients who are being treated with PLM, more over RO generation is probably enahnced by locally generated cytokines. In fact, PLM enhances cytokine release from PMN, lymphocytes, monocytes, and endothelial cells (in press[33]). Fibroblast proliferation is probably closely associated with the inflammatory reactions in the lung. Fibroblasts separated from the human gingiva and rabbit pulmonary tissue more proliferated by the addition of PLM and the supernatants of PLM-treated human mononuclear leukocytes (in press[33]). These investigations indicate that PLM impairs the pulmonary tissue by inducing the generation of ROS.

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