PEPLOMYCIN SULFATE AND PULMONARY FIBROSIS: HYDROXYPROLINE, URONIC ACID, PROLINE HYDROXYLASE AND GLUCOSAMINE 6-PHOSPHATE SYNTHETASE IN LUNGS OF HAMSTERS TREATED WITH PEPLOMYCIN

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(Received for publication January 28, 1983)

Effect of peplomycin sulfate (PLM) on pulmonary fibrosis was examined. Hydroxyproline, uronic acid, proline hydroxylase (EC 1.14.11.2) and glucosamine 6-phosphate synthetase (EC 2.6.1.16) in lungs of hamsters treated with PLM were studied and compared with those of hamsters treated with bleomycin (BLM).

PLM, when administered intraperitoneally, one injection daily for 10 consecutive days, at either a high- (5 mg/kg) or low- (2.8 mg/kg) dosage-level, caused no significant increase of lung hydroxyproline and uronic acid as compared with controls. BLM on the other hand effected a significant increase in lung hydroxyproline on the high-dosage level (5 mg/kg) but not on the low-dosage level (2.8 mg/kg).

In contrast, when administering PLM intratracheally, the concentrations of hydroxyproline in lungs increased 20% over the control levels. A transient increase of proline hydroxylase and glucosamine 6-phosphate synthetase also occurred shortly after the instillation. These increases were also observed in the corresponding groups treated with BLM, which confirmed the previous observations by other investigators. However, the magnitude of the increase was relatively lower in those values of PLM as compared with those of BLM.

These data suggested that (1) PLM, when administered with multiple dosages intraperitoneally, showed no significant effect on the elevation of lung hydroxyproline; (2) PLM, when administered with a dose intratracheally, induced pulmonary fibrosis similar to that caused by BLM. However, the hydroxyproline accumulation in lungs of PLM-treated hamsters was less than in those of the BLM-treated; (3) The fibrotic effect on the lungs caused by either PLM or BLM was probably attributed to acceleration of the syntheses of collagen and acidic glycosaminoglycans.

Bleomycin (BLM) has highly effective anti-neoplastic activity which has been used to control epidermal carcinoma[1,2] and HODGKIN's disease[3], as well as cancer of the testis[4]. Adversely, BLM has also been known to induce pulmonary fibrosis which is a dose-related disorder and often leads to fatal consequences[5].

Recently, peplomycin sulfate (PLM), a new derivative of BLM has been prepared and proven to have a less fibrotic activity while retaining an effective anti-neoplastic action similar to that of BLM[6].

This paper reports the effect of PLM as compared with that of BLM, on lungs of hamsters. Collagen metabolism in lungs was studied by measuring hydroxyproline and the activity of proline hydroxylase (EC 1.14.11.2). Acidic glycosaminoglycan metabolism in lungs was also investigated by measuring uronic acid and the activity of glucosamine 6-phosphate synthetase (EC 2.6.1.16). The effects of

PLM as well as BLM by the different routes of administration were also examined.

Materials and Methods

Syrian golden hamsters (Tokyo Experimental Animals, Inc.), 250 males weighing 110±5 g, were used for the experiments. Bleomycin (BLM) and peplomycin sulfate (PLM) were obtained from Nippon Kayaku Company, Ltd., Tokyo.

BLM or PLM was dissolved in physiological saline and the amount administered at two different dosage-levels on each injection, 2.8 mg or 5.0 mg per kg of body weight. For intratracheal administration (i.t.), a single injection was carried out at each dosage; the animals were sacrificed at different intervals for the experiments. For the intraperitoneal administration (i.p.), in 0.3 ml of saline containing PLM or BLM was injected at either the high- or low-dosage level, and the injection was carried out daily for 10 consecutive days, (totally 10 injections on each hamster). For controls, 0.3 ml of saline was injected.

Intratracheal Administration

The hamster was lightly anesthetized by injecting Nembutal intraperitoneally. The trachea was exposed after making a small incision on the neck and separating the trap muscles; then 0.3 ml of the respective dosage level of PLM or BLM was instilled; normal saline was given to the control.

Lung Homogenates

Hamsters were sacrificed and exsanguinated by cutting renal arteries under Nembutal anesthesia. Lung were removed, separated from the trachea and large blood vessels and the lung tissue weighed. The tissue was then minced to *ca.* 2 mm sections. An aliquot (*ca.* 100 mg) was homogenized in a POTTER's Teflon homogenizer with 9 volumes of 50 mM Tris-HCl buffer, pH 7.6, containing 0.1 mM of dithiothreitol, and the homogenate was centrifuged at 35,000×*g* for 20 minutes. Aliquots of the supernatant were used for assaying proline hydroxylase and glucosamine 6-phosphate synthetase. The remaining lung tissue was dehydrated and defatted with 10 volumes of acetone and weighed. Aliquots of the dry-defatted lung tissue were used for assaying hydroxyproline and uronic acid.

Determination of Hydroxyproline

Aliquots of the dry-defatted lung tissue were hydrolyzed with 1 ml of 6 N HCl in a sealed vacuum test tube at 110°C for 18 hours. Excess HCl was evaporated and hydroxyproline was determined according to the method of WOESSNER[7].

Determination of Uronic Acid

Aliquots of the dry-defatted lung tissue (*ca.* 10 mg each) were suspended in 0.5 ml of 0.1 M Tris-HCl buffer solution, pH 7.8 containing 5 mM of $CaCl_2$, and subsequently digested with pronase E (Kaken Chem. Inc.) for 48 hours at 40°C. Then trichloroacetic acid (TCA) was added to the enzyme hydrolysate to give a final concentration of 10% and the mixture was centrifuged at 5,000×*g* for 10 minutes. The enzyme hydrolysate was subsequently neutralized with NaOH and dialyzed against running water overnight; ethanol (3 volumes containing potassium acetate at 1%) was added and the solution was kept overnight at 2~3°C. The precipitate obtained by centrifugation was dissolved in 1 ml distilled water, and the uronic acid content in the sample was determined according to the method of BITTER and MUIR[8].

Measurement of Proline Hydroxylase in Lung

The assay was carried out according to the method of PETERKOFSKY and DIBLASIO using [^{3}H]protocollagen as substrate[9]. Briefly, the enzyme activity was measured in the mixture of 15 μl of 1-[3,4-^{3}H]-proline-labelled protocollagen, the lung extract and the buffer solution. The final volume was made to 100 μl with the buffer solution, which consisted of Tris-HCl, pH 7.6, 40 mM; sodium ascorbate, 1.0 mM; α-ketoglutarate, 1.0 mM; ferrous ammonium sulfate, 0.2 mM; catalase, 0.4 mg/ml; bovine serum albumin, 2.0 mg/ml; and dithiothreitol, 0.5 mM. The reaction mixture was incubated for 15 minutes at 37°C; 0.4 ml of 6.25% TCA was added. To this mixture, 15 μl of 2.5% solution of serum albumin was added as a carrier, the solution was centrifuged for 5 minutes at 1,500×*g*, and the supernatant was then removed. The precipitate was washed with 0.5 ml of 5% TCA, and again the

supernatant was removed by centrifugation. The combined supernatant was passed through Bio-Rad resin AG 50W-X8 column (0.5×2 cm); the effluent was collected in a liquid scintillation vial, scintillation fluid (Packard Insta-Gel) was added, and radioactivity was counted. Under this experimental condition, our preliminary result showed that the enzyme activities (radioactivity released in the supernatant) had a linear response with the increasing amounts of the lung homogenate obtained from a normal hamster. This assay system also had a linear relationship up to 20 minutes incubation.

Measurement of Glucosamine 6-Phosphate Synthetase in Lung

The enzyme activity was measured according to the method of POGELL and GRYDER[10]. Briefly, to the supernatant fraction of lung homogenate 1 ml of the substrate solution was added. The substrate solution consisted of 40 μl phosphate buffer, pH 7.5; 10 μM of reduced glutathione; 1 μM of EDTA; 15 μM of L-glutamine; and 10 μM fructose-6-phosphate. After the mixture had been incubated for 1 hour at 37°C, 1 ml of 0.4 N TCA was added, and the mixture was then centrifuged for 10 minutes at 5,000×g. The amount of hexosamine 6-phosphate synthesized in the supernatant was measured by the procedure of BLIX[11]. One unit of the synthetase was defined as the amount of enzyme which could synthesize one nmol of hexosamine 6-phosphate per minute. Protein in the supernatant was determined by the method of LOWRY *et al.*[12].

Results

Mortality

Most of the animals survived after the multiple i.p. injections of PLM or BLM, except in the high-dosage BLM group, from which one out of fifteen died. In contrast, PLM, when administered with a single i.t. dose, was apparently toxic: the mortality rate was 53% and 10% for the high- and low-dosage group, respectively (Table 1). Interestingly, the deaths occurred mostly in the period between 4 to 16 days after the instillation. The i.t. administration of BLM also showed high mortality in both high- and low-dosage groups: 53 and 37%, respectively.

Table 1. Effect of PLM and BLM on mortality of hamsters.

Route	i.t.				i.p.			
Dosage	2.8 mg/kg		5.0 mg/kg		2.8 mg/kg		5.0 mg/kg	
	Rate	%	Rate	%	Rate	%	Rate	%
PLM	3/30	10	8/15	53	0/15	0	0/15	0
BLM	11/30	37	8/15	53	0/15	0	1/15	6
Control	1/25	4	——		0/20	0	——	

Mortality rate was expressed as number of deaths within the first 20 days per number of hamsters tested. PLM and BLM were dissolved in 0.3 ml of saline and intratracheally instilled or intraperitoneally injected. Control animals were instilled with 0.3 ml of physiological saline.

Change of Body Weight

The gain of body weight of the hamsters treated by i.p. with PLM was slightly depressed as compared with that of the control (Fig. 1). The change of body weight of PLM groups was relatively less as compared to that of the BLM. However, when the animals were treated by the i.t. route, body weight decreased in all the groups following a single instillation, and this decrease was found to be especially profound in those given BLM. The group injected with the low-dosage of PLM lost body weight slightly 5 days after the treatment, but recovered on day 10, and thereafter, resumed gaining. For the animals receiving the high-dosage of PLM, the resumption of gaining occurred after day 15.

Lung Weight

Change of lung weight of the animals treated by i.p. with PLM was not significantly different from that of the control (Fig. 2). However, when administered by i.t., lung weight of the animals which received PLM increased gradually and reached a peak at day 10, thereafter declining to that of the control. The intraperitoneally BLM-treated animals also showed significant elevation by day 45.

Fig. 1. Effect of PLM and BLM, change of body weight.

Each point represents the mean±standard error of mean, and parentheses are the number of animals used.

Control ○, PLM (2.8 mg/kg) —▲—, PLM (5 mg/kg) ---▲---, BLM (2.8 mg/kg) —●—, BLM (5 mg/kg) ---●---.

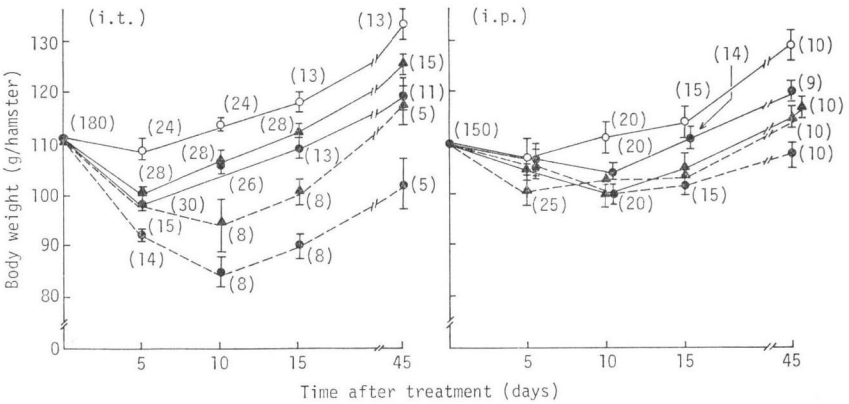


Fig. 2. Effect of PLM and BLM, change of dry, defatted lung weight following the intratracheal or intraperitoneal administration.

Each point represents the mean±standard error of mean from the experiments of 4 to 8 animals on each group.

* $P<0.05$; ** $P<0.01$

Control ○, PLM (2.8 mg/kg) ▲, BLM (2.8 mg/kg) ●.

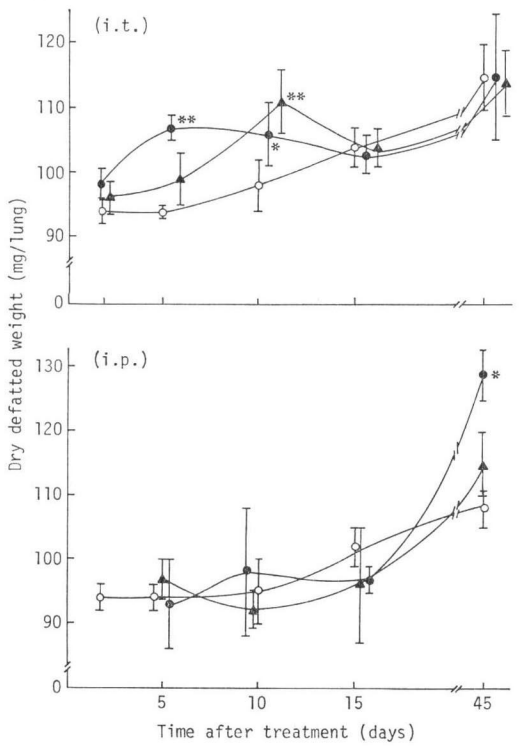
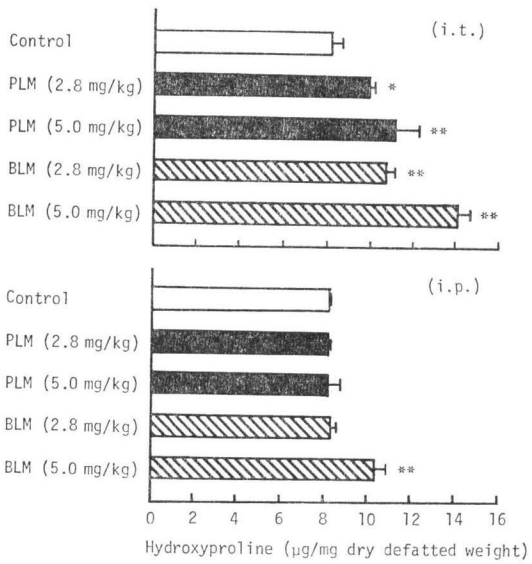


Fig. 3. Hydroxyproline contents of lungs from the animals treated intraperitoneally or intratracheally with PLM of BLM.

The data were obtained from the hamsters 45 days after the experiments. The data represent the mean±standard error of mean from the experiments of 3 to 6 hamsters on each group.

* $P<0.05$; ** $P<0.01$



Hydroxyproline Content in Lungs

The hydroxyproline content of lungs in the animals treated with PLM at both high- and low-dosage i.p. showed no significant change,

Table 2. Total hydroxyproline in whole lung (45 days post-treated).

	(mg/kg)	Dry weight (mg) / Lung	Hydroxyproline / mg dry tissue	Total hydroxyproline
i.t.	Control	115± 5	8.2±0.5	943± 55
	PLM (2.8)	114± 5	9.9±0.3*	1,129± 81*
	PLM (5.0)	121±13	11.2±1.1**	1,350±202**
	BLM (2.8)	115±11	10.8±0.4**	1,242± 97**
	BLM (5.0)	117± 4	14.1±0.6**	1,665±126**
i.p.	Control	108± 3	8.2±0.3	905± 30
	PLM (2.8)	113± 5	8.2±0.2	927± 24
	PLM (5.0)	124± 6	8.2±0.6	1,007± 39
	BLM (2.8)	129± 4	8.3±0.3	1,071± 57*
	BLM (5.0)	113± 2	10.3±0.6**	1,164± 63**

* $P<0.05$; ** $P<0.01$.

Fig. 4. Changes of hydroxyproline contents in lungs from the animals treated intratracheally with PLM or BLM.

Each point represents the mean±standard error of mean from the experiments of 3 to 5 hamsters on each group. *$P<0.05$; **$P<0.01$. Control ○, PLM (2.8 mg/kg) ▲, BLM (2.8 mg/kg) ●.

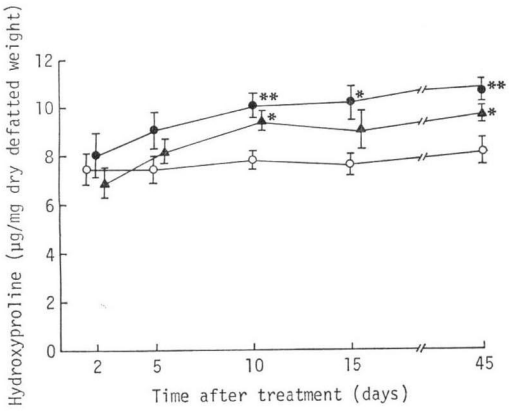
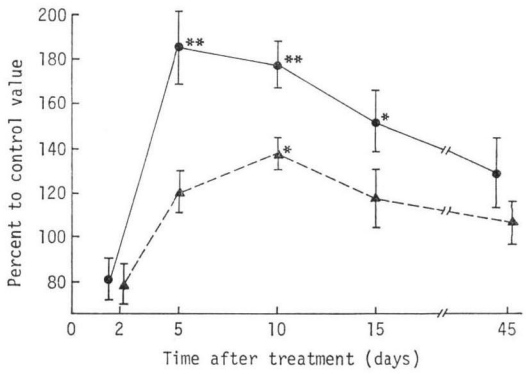


Fig. 5. Change of proline hydroxylase levels in lungs of the animals treated intratracheally with PLM or BLM.

Each point represents the mean±standard error of mean from the experiments of 4 hamsters on each group. *$P<0.05$; **$P<0.01$.

PLM (2.8 mg/kg) ▲, BLM (2.8 mg/kg) ●.



whereas the BLM group showed a significant elevation at high- ($P<0.01$) dosage. In contrast, by the i.t. route, all the lungs treated with PLM or BLM showed elevation of hydroxyproline: 45 days after the treatments, values were found to exceed the hydroxyproline level of the control by 20, 43, 32 and 77% for PLM 2.8 mg/kg, PLM 5.0 mg/kg, BLM 2.8 mg/kg and BLM 5 mg/kg, respectively (Fig. 3, Table 2). A significant elevation of the hydroxyproline content occurred 10 days after the i.t. administration of PLM or BLM (Fig. 4); thereafter the level of elevation remained higher than that of the corresponding control.

Proline Hydroxylase

A transient elevation of proline hydroxylase was observed; the enzyme activity in the lungs rapidly rose within 5 days after the i.t. administration of PLM or BLM; thereafter the increase gradually diminished and reached the control level after 45 days (Fig. 5). The elevation of the enzyme activity was much

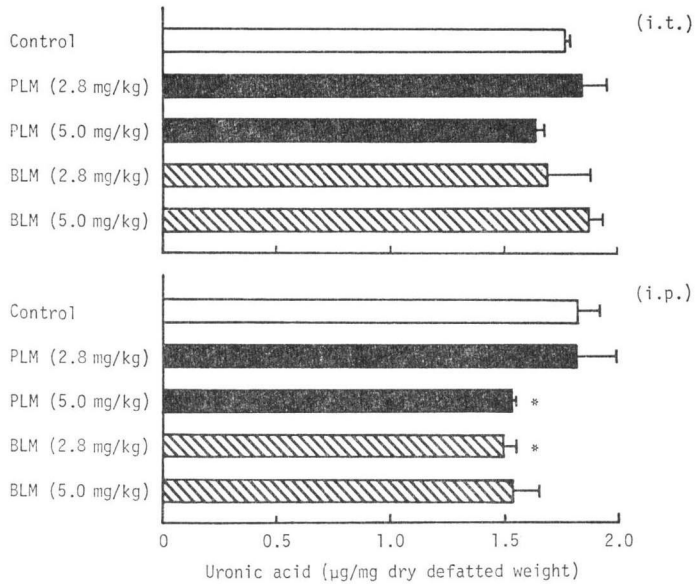
Table 3. Total uronic acid in whole lung (45 days post-treated).

(mg/kg)	Dry weight (mg) / Lung	Uronic acid (μg) / mg dry tissue	Total uronic acid (μg)
i.t. Control	115± 5	1.77±0.14	204±20
PLM (2.8)	114± 5	1.80±0.12	205± 3
PLM (5.0)	121±13	1.64±0.12	198±19
BLM (2.8)	115±11	1.69±0.19	194±35
BLM (5.0)	117± 4	1.87±0.06	219± 8
i.p. Control	108± 3	1.82±0.11	197±13
PLM (2.8)	113± 5	1.82±0.10	206± 9
PLM (5.0)	124± 6	1.53±0.02*	190± 9
BLM (2.8)	129± 4	1.50±0.05*	194± 8
BLM (5.0)	113± 2	1.54±0.11	174±11

* $P<0.05$.

Fig. 6. Uronic acid contents in lungs of the animals treated intraperitoneally and intratracheally with PLM and BLM.

The data were obtained from the animals 45 days after the experiments. The data represent the mean±standard error of mean from the experiments of 3 to 6 animals on each group. *$P<0.05$.



higher in the lungs treated with BLM than those with PLM; the value at the peak was in excess of the control value by 80 and 30%, respectively.

Uronic Acid Contents in Lungs

There was no significant change in the total uronic acid content in lungs of the hamsters treated with PLM or BLM administered by either the i.p. or the i.t. route (Table 3). In the groups treated with PLM 5.0 mg/kg or BLM 2.8 mg/kg, i.p., the uronic acid content per mg of the dry defatted lung tissue significantly decreased after 45 days (Fig. 6).

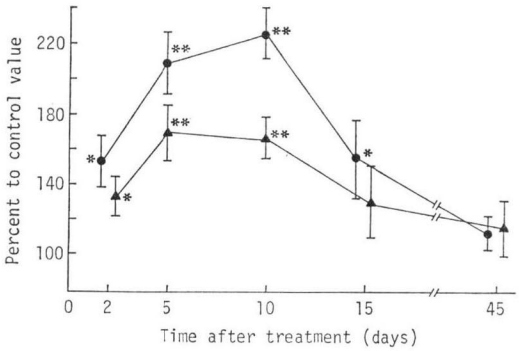
Glucosamine 6-Phosphate Synthetase

There was a transient elevation of glucosamine 6-phosphate synthetase in lungs following the i.t. administration of PLM 2.8 mg/kg and BLM 2.8 mg/kg. This elevation was observed in both groups 2 days after the administration and peaked at the 10th day, thereafter gradually diminishing to the control level (Fig. 7). The elevation of the enzyme was higher in the groups of BLM-treated hamsters.

Fig. 7. Change of glucosamine 6-phosphate synthetase in lungs of the animals treated intratracheally with PLM or BLM.

Each point represents the mean±standard error of mean from the experiments of 4 animals on each group. $*P<0.05$; $**P<0.01$.

PLM (2.8 mg/kg) ▲, BLM (2.8 mg/kg) ●.



Discussion

BLM is a mixture consisting of mainly BLM A2 and B2 in the ratio of about 75: 25.[13] PLM is a derivative of BLM which contains *N*-(3-aminopropyl)-α-(*S*)-phenylethylamine as the terminal amine[6]. The compound has been developed as a broad spectrum antineoplastic drug and has an effectiveness similar to the parent compound, BLM. BLM and its derivatives are known to have a potent antineoplastic activity, and its cytotoxic action has been well described by various workers[14~21]. BLM shows a serious side-reaction, leading to fibrotic lung disease. This disorder can probably be attributed to the chronic toxic effect of BLM on the lung, since (a) BLM has a high affinity toward the tissue of lungs (also skin) as compared with other organs[22], and (b) the BLM-inactivating enzyme system was relatively lower in the tissue of lung (or skin) in comparison to other tissues[23]. In order to minimize the side-effect while retaining the anti-neoplastic activity of the drug, PLM has been developed. Our present studies reveal that collagen accumulation in the lungs, which was quantified by measuring the hydroxyproline content, was not greatly excess in the hamsters treated with PLM by the intraperitoneal route as compared with those treated with BLM. However, the direct i.t. instillation of PLM into the lungs induced a slight elevation of collagen in the lungs. In contrast, BLM showed a striking effect of collagen accumulation and the elevation of proline hydroxylase in the lungs, and our present observations on BLM support those reported by previous investigators[24~29].

The increase of acidic glycosaminoglycans (AGAG) was often accompanied by the elevation of lung collagen in pulmonary fibrosis[30,31]. OTSUKA *et al.* previously showed that BLM stimulates the synthesis of AGAG in fibroblast cell culture[30] and CANTOR *et al.* reported that the synthesis of AGAG was accelerated in fibrotic lungs of animals treated with BLM[31]. Recently, we have observed the elevation of glucosamine 6-phosphate synthetase in the lungs of hamsters with the BLM treatment[29]. This elevation of the enzyme activity was followed with the increase of AGAG in the lung. It has also been believed that $AGAG_s$, especially chondroitin sulfate proteoglycan, play an important role in the synthesis of collagen fibers[32~34]. Furthermore, it has been suggested that $AGAG_s$ may provide the initial matrix for collagenous tissues, since studies of wound healing showed that the deposit of $AGAG_s$ preceded the accumulation of collagen[35].

In our present study, both the elevation of proline hydroxylase, which is an important factor in controlling the rate of synthesis of collagen[36,37], and the activation of glucosamine 6-phosphate synthetase, which is considered as a rate limiting step for the synthesis of $AGAG_s$[38], were observed prior to the increase of collagen in the lungs. Fibrotic lung diseases are commonly caused by a chronic alveolitis which leads to derangement of the alveolar structure including parenchymal and interstitial tissues; subsequently this process leads to the loss of the functional units of the lungs[39]. Although the pathogenesis of fibrosis caused by BLM is unknown, the affected lungs manifest themselves with edema, hemorrhage and infiltration of monocytes and polymorphonuclear leukocytes[40]. We observed a similar reaction in the hamsters treated with BLM and some of the animals treated with PLM. Interestingly, our studies also

revealed that the days of the death coincided with the time of the maximum inflammation which occurred mostly in the period between 5 to 15 days (Fig. 2). It is noteworthy that the time of maximum inflammation in the PLM i.t.-instilled hamsters appeared later (11 days) than that of the corresponding animals treated with BLM (5 days). Incidentally, the former group had less collagen in the lungs as compared to that of the BLM group.

It is most interesting to observe a negligible fibrotic reaction in those hamsters treated by i.p. with PLM, even when administered with the daily high dosage (5 mg/kg) for 10 days consecutively. In a similar experiment using mice, EBIHARA *et al.* reported that pulmonary fibrosis was observed at the same dosage (5 mg/kg)[41]. There is no explanation for this discrepancy but is probably due to the difference in species of the animal used.

Our data showed a less or minimum pulmonary toxicity of PLM, especially when administered by i.p., as compared with that of BLM.

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