Studies on Medicinal Resources from Livestock. II. Anti-allergic Effects of Pig Bile.[1,2] (2)

Tatsuyoshi NAKAGAMI,* Non YAMASAKI and Shiro TAJI

R & D Center, Nippon Meat Packers Inc., 451, Takahata, Hiraoka-cho, Kakogawa, Hyogo 675-01, Japan. *Received August 24, 1989*

Anti-allergic activities of lyophilized pig bile ([PB]) were examined in mice with picryl chloride-induced contact dermatitis (PC-CD), an experimental model of delayed-type hypersensitivity (DTH; type-IV allergy). PC-CD was markedly inhibited by an oral administration of [PB] within 4 h after but not during 8 to 16 h after challenge with picryl chloride.

Anti-inflammatory activities of [PB] were also examined in acetic acid-induced mouse increased vascular permeability, hypotonic-hyperthermic lysis of rat erythrocytes and carrageenin-induced rat hind paw edema. [PB] had no effect on these models. The present study suggests that [PB] inhibits PC-CD through its immuno-modulation in the inductive phase of DTH rather than by an anti-inflammatory action.

Keywords anti-allergic effect; pig bile; delayed type hypersensitivity; picryl chloride induced contact dermatitis; anti-inflammatory effect

Recent Chinese reports[3,4] on traditional Chinese medicine show that animal biles can be used for therapy of bronchitis, asthma and hypersensitivities. Since these diseases are associated with various allergic reactions, the anti-allergic effects of animal biles were examined through some experimental allergic disease models in the previous study.[1] It was found that pig bile ([PB]) markedly prevented picryl chloride-induced contact dermatitis (PC-CD) in mice and sheep red blood cells (SRBC)-induced footpad swelling in mice, both of which are experimental models of delayed-type hypersensitivity (DTH; type-IV allergy). In order to study the mode of action of [PB], the effects of various treatment regimens with [PB] on PC-CD inhibition and anti-inflammatory effects in some experimental models were examined in the present study.

Experimental

Materials Biles of pigs (triple crosses among Large Yorkshire, Landrace, Duroc and Hampshire strains) were collected from gallbladders with a sterile plastic syringe, pooled and lyophilized ([PB]). The result of chemical analysis of [PB] was reported in the previous paper.[1]

Animals: Male Wistar rats and male ddY mice were used. They were housed in an air conditioned room with a commercial chow (Oriental Yeast Co., Ltd.) and tap water *ad libitum*.

Methods 1) Effects of Various Treatment Regimens with [PB] on PC-CD Inhibition PC-CD was induced according to the method of Asherson and Dtak.[5] Male ddY mice weighing 18 to 22 g were sensitized by applying 0.1 ml of 7% picryl chloride (PC) in ethanol to their abdominal skins which had been shaved on the previous day. After 7 d, contact dermatitis was induced by applying 0.02 ml of 1% PC in olive oil to both ear lobes of the mice. After a further 3 d, the mice were resensitized with 7% PC in ethanol. Seven days after resensitization, the mice were challenged with 1% PC in olive oil to induce contact dermatitis again (challenge). Each test drug, suspended in distilled water, was orally administered before and/or after challenge with 1% PC. Prednisolone (Sigma Chem. Co.) was used as a reference standard. Mice in the control group were orally given distilled water in place of a drug suspension. Ear thickness was measured with a dial thickness gauge (Ozaki Co.) immediately before (B) and 24 h after challenge (A). Ear swelling (%) was calculated from the mean value of (A) and (B) by using the following equation;

$$\text{ear swelling } (\%) = (A/B - 1) \times 100$$

Inhibition rate (%) was calculated from ear swelling (%) of the control group (C) and that of each test drug group (D) by using the following equation;

$$\text{inhibition } (\%) = (1 - D/C) \times 100$$

Immediately after the final measurement of ear swelling, their spleens of mice were removed to measure their wet weights.

a) Oral Administrations Immediately before and/or 16 h after Challenge: Each drug suspension was orally administered immediately before, or 16 h after, or both immediately before and 16 h after challenge.

b) Oral Administrations during 16 h after Challenge: Each drug suspension was orally given 0, 4, 8, 12 or 16 h after challenge.

2) Anti-inflammatory Effects a) Effect of Increased Vascular Permeability Induced by Acetic Acid in Mice: Male ddY mice weighing around 20 g were fasted overnight. Indomethacin (Sigma Chem. Co.) was used as a reference standard. [PB] and indomethacin, suspended in distilled water, were orally administered 30 min before the i.v. injection of 4% pontamine sky blue (PSB) solution in saline (0.1 ml/10 g body weight). With mice in the control group, distilled water was orally administered in place of a drug suspension. Five min after the injection of PSB, 0.6% acetic acid solution in saline was injected intraperitoneally (0.1 ml/10 g body weight) to induce increased vascular permeability according to the method of Kostar *et al.*[6] After 20 min, the mice were killed by decapitation. Distilled water was injected intraperitoneally (10 ml/animal) and fluid in the peritoneal cavity was collected after a gentle 30-s massage of the abdomen. The fluid was centrifuged at 3000 rpm for 10 min with 0.1 ml of 0.1 N NaOH to remove protein. The absorbance of the supernatant was measured at 590 nm to calculate inhibition (%) to the control group.

b) Effect of Hypotonic-Hyperthermic Lysis of Rat Erythrocytes: The following experiments were carried out according to the method of Glenn and Bowman.[7]

1. Hypotonic-Hyperthermic Lysis: A 10% rat erythrocyte suspension was prepared by centrifuging freshly obtained heparinized blood of male Wistar rats and adding 0.15 M phosphate buffer (pH 7.4). A 3.0 ml portion of 0.015 M phosphate buffer containing each drug at the designated concentration was added to 3.0 ml of the erythrocyte suspension. In order to produce 100% hemolysis, 0.1% $NaCO_3$ solution in distilled water was added to the erythrocyte suspension in place of 0.015 M phosphate buffer. The mixture was heated at 53 °C for 20 min in a water bath, chilled for 5 min in an ice bath and centrifuged. The absorbance of the supernatant was measured at 540 nm to determine hemolysis (%). Sodium citrate was used as a reference standard. Each assay was carried out in triplicate.

2. Hypotonic Lysis: The mixture was heated at 37 °C for 1 h (instead of 53 °C for 20 min) in the experiment described in 1.

3. Hyperthermic Lysis: A 3.0 ml portion of 0.15 M phosphate buffer (instead of 0.015 M phosphate buffer) containing each drug was added to 3.0 ml of the erythrocyte suspension in the experiment described in 1.

c) Effect on Carrageenin-Induced Hind Paw Edema in Rats: The experiment was carried out by the method of Winter *et al.*[8] Male Wistar rats weighing 120 to 140 g were fasted overnight. Indomethacin (Sigma Chem. Co.) was used as a reference standard. [PB] and indomethacin, suspended in distilled water, were orally administered. With rats in the control group, distilled water was orally given in place of a drug suspension. At 1 h after the oral administration of the test drug, 0.1 ml of 1.0% carrageenin solution in sterile saline was injected subcutaneously into the plantar surface of the right hind paw of each rat. Hind paw volumes were measured by the displacement method in a water bath at every 1 h from 0 to 5 h after the injection of carrageenin. Increase (%) in hind paw volume was calculated by using the following equation:

increase (%) $= (A/B - 1) \times 100$

A: Hind paw volume at every 1 h after the injection.
B: Hind paw volume at 0 h (immediately) after the injection.

3) Statistical Analysis All data are mean values ± S.E. Statistical analysis was performed by Yamazaki's ASSIT method[9] (based on Dunnet's[10,11] and Scheffe's methods[12]).

Results

1) Effects of Various Treatment Regimens with [PB] on PC-CD Inhibition a) Oral Administrations Immediately before and/or in 16 h after Challenge Figure 1 shows the results of one or two treatments with [PB]. An effective PC-CD inhibition was observed when [PB] was orally administered immediately before or immediately before and 16 h after challenge with PC. However no PC-CD inhibition was observed when it was administered 16 h after challenge. Prednisolone markedly inhibited PC-CD whenever it was administered. There was no significant difference in spleen weight between the control and the treated groups.

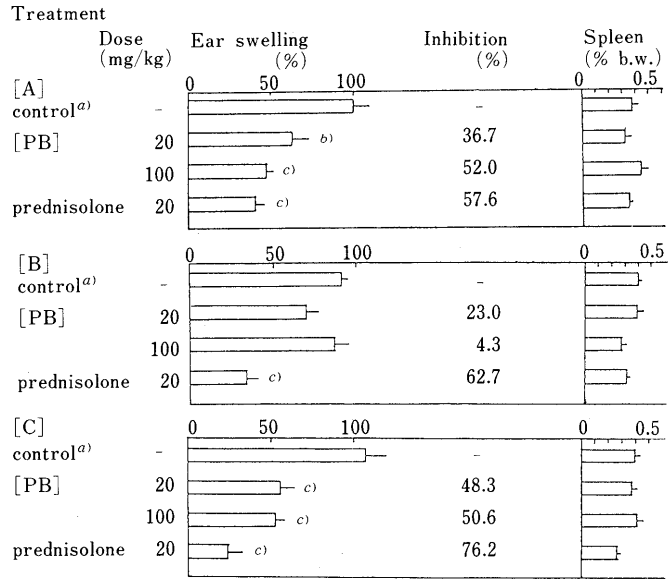

Fig. 1. Influence of One or Two Treatments with [PB] on PC-CD Inhibition in Mice

[A], [B] and [C] indicate the groups orally given [PB] immediately before (0 h), 16 h after, and 0 and 16 h after challenge with PC, respectively. Each group consisted of 10 animals (mean ± S.E.). *a*) Water was orally administered at 20 ml/kg. *b*, *c*) Significantly different from the control group at $p < 0.05$ and $p < 0.01$, respectively.

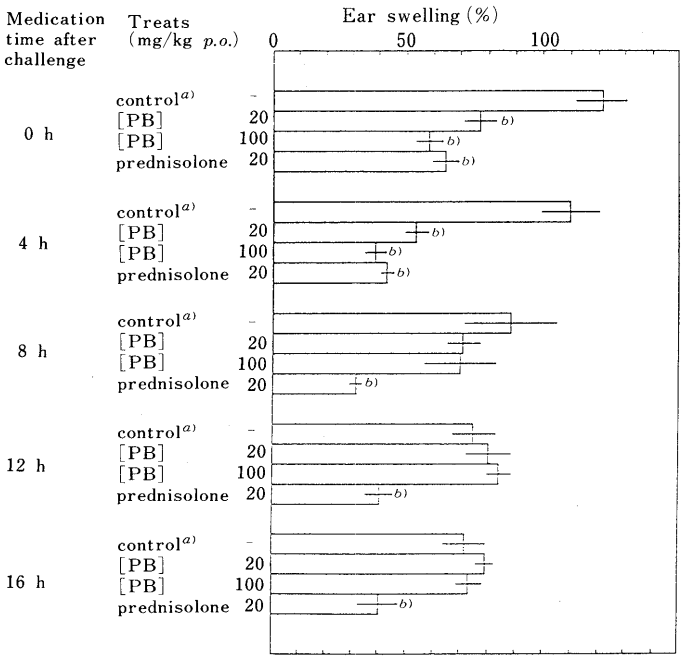

Fig. 2. Influence of Treatment with [PB] during 16 h after Challenge on PC-CD Inhibition in Mice

Each test drug was administered immediately before (0), 4, 8, 12 or 16 h after challenge with PC. Each group consisted of 10 animals (mean ± S.E.). *a*) Water was administered at 20 ml/kg. *b*) Significantly different from the control group at $p < 0.01$.

b) Oral Administrations during 16 h after Challenge Figure 2 indicates the results of treatments with [PB] during 16 h after challenge with PC. [PB] inhibited PC-CD effectively when orally administered within 4 h after, but not 8 to 16 h after challenge. Prednisolone markedly inhibited PC-CD whenever orally administered.

2) Anti-inflammatory Activities of [PB] Increased vas-

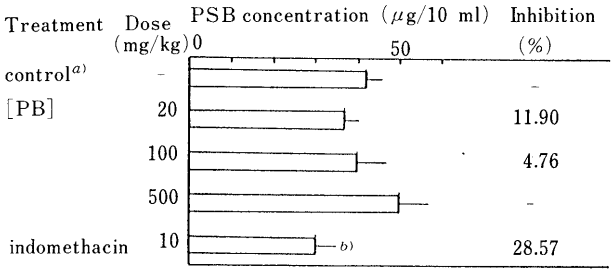

Fig. 3. Effect of [PB] on Increased Vascular Permeability by Acetic Acid in Mice

Each test drug was orally administered to mice 30 min before the intravenous injection of PSB. Five min after the PSB injection, acetic acid injected intraperitoneally. Each group consisted of 10 animals (mean ± S.E.). *a*) Water was orally administered at 20 ml/kg. *b*) Significantly different from the control group at $p < 0.05$.

TABLE I. Effect of [PB] on Hypotonic-Hyperthermic Lysis of Rat Erythrocytes

Treatment	Concentration (μg/ml)	Hemolysis (%)
Control	—	97.0 ± 0.8
[PB]	100	99.3 ± 0.6
	500	99.8 ± 0.8
Sodium citrate	1000	95.4 ± 0.5
	5000	49.0 ± 0.2

Each value represents the mean ± S.E. of 6 tubes.

TABLE II. Effect of [PB] on Hypotonic Lysis of Rat Erythrocytes

Treatment	Concentration (μg/ml)	Hemolysis (%)
Control	—	80.4 ± 0.6
[PB]	100	73.0 ± 0.5
	500	78.9 ± 0.2
Sodium citrate	1000	45.5 ± 0.2
	5000	3.1 ± 0.2

Each value represents the mean ± S.E. of 6 tubes.

TABLE III. Effect of [PB] on Hyperthermic Lysis of Rat Erythrocytes

Treatment	Concentration (μg/ml)	Hemolysis (%)
Control	—	60.9 ± 1.4
[PB]	100	40.7 ± 0.5
	500	96.5 ± 0.2
Sodium citrate	1000	50.7 ± 0.1
	5000	38.7 ± 0.6

Each value represents the mean ± S.E. of 6 tubes.

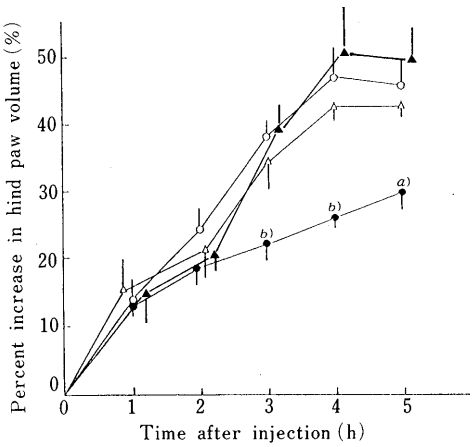

Fig. 4. Effect of [PB] on Carrageenin-Induced Hind Paw Edema in Rats

Rats were injected with carrageenin subcutaneously 1 h after oral administration of the test drug. Each group consisted of 10 animals (mean ± S.E.). —○—, control water 2 ml/kg *p.o.*; —▲—, [PB] 200 mg/kg *p.o.*; —△—, [PB] 500 mg/kg *p.o.*; —●—, indomethacin 10 mg/kg *p.o.* *a*, *b*) Significantly different from the control group at $p<0.05$ and $p<0.01$, respectively.

cular permeability induced by acetic acid was inhibited significantly by oral administration of indomethacin to mice at a dose of 10 mg/kg, but not by [PB] at the doses tested (Fig. 3).

The inhibitory effect of [PB] on hypotonic-hyperthermic lysis of rat erythrocytes was examined at concentrations of 100 and 500 μg/ml, while [PB] at concentrations of more than 500 μg/ml interfered with the measurement of hemolysis due to its bile pigment. Table I indicates that [PB] did not inhibit hypotonic-hyperthermic lysis of rat erythrocytes at the given concentrations. Effects of [PB] on hypotonic and hyperthermic lysis are shown in Tables II and III, respectively. Lysis was slightly inhibited in both models, but was not dependent on the concentration of [PB]. Sodium citrate inhibited lysis at concentrations of 1000 and 5000 μg/ml.

The inhibitory effect of [PB] on rat hind paw edema induced by carrageenin was examined (Fig. 4). Indomethacin inhibited the edema at a dose of 10 mg/kg (*p.o.*) 3, 4 and 5 h after the injection of carrageenin, but [PB] did not even at doses of 200 and 500 mg/kg (*p.o.*).

Discussion

In order to find medicinal resources from livestock, the authors first examined the anti-allergic activities of animal biles and reported that [PB] markedly inhibited experimental models of DTH.[1] In the present study, the anti-allergic and anti-inflammatory activities of [PB] were further examined to elucidate the mode of action of [PB].

To examine the effects of various treatment regimens with [PB] on PC-CD inhibition, [PB] was orally administered immediately before and/or 16 h after challenge with PC. [PB] showed no effect on PC-CD inhibition when administered once 16 h after challenge. However, a strong inhibition was observed when [PB] was administered once immediately before and twice immediately before and 16 h after challenge. These results indicate that only the administration of [PB] immediately before challenge had an inhibitory effect on PC-CD. Further, the inhibitory activities of [PB] for PC-CD were studied by oral administration at 0, 4, 8, 12 or 16 h after challenge. Oral administration of [PB] within 4 h after challenge inhibited PC-CD, but that during 8 to 16 h did not. Prednisolone, a steroid drug, strongly inhibited PC-CD whenever orally administered. DTH is thought to be caused by two main phases, the inductive phase (up to 14 to 16 h after challenge with PC) and the effector phase (appeared more than 16 h after challenge). The above findings suggest that [PB] has an inhibitory effect on the inductive phase of DTH.

The anti-inflammatory activities of [PB] were examined in three experimental models, increased vascular permeability induced by acetic acid in mice, hypotonic-hyperthermic lysis of rat erythrocytes and rat hind paw edema induced by carrageenin. [PB] showed no significant inhibitory activities in these models.

The present study strongly suggests that [PB] inhibits PC-CD through immuno-modulation at the inductive phase of DTH rather than by anti-inflammatory action. Further studies are in progress on the mode of action and on the active components in pig bile.

Acknowledgments We thank Professor Michinori Kubo (Kinki University), Dr. Shigeru Morita (Osaka City Institute of Public Health and Environmental Sciences), Dr. Tamotsu Shigehisa (R & D Center of Nippon Meat Packers Inc.) and Professor Toshiomi Yoshida (Osaka University) for helpful suggestions and critical review of the manuscript.

References and Notes

1) Part I: M. Kubo, T. Nakagami, N. Yamasaki, M. Ito, M. Nogami and S. Taji, *Chem. Pharm. Bull.*, **37**, 3409 (1989).
2) Part of this paper was presented at the 106th Annual Meeting of the Pharmaceutical Society of Japan, Chiba, April, 1986.
3) S. Gao, D. Dai, Q. Fan and M. Deng, "Chang Jian Yao Yong Dong Wu," Shanghai Ke Xue Ji Shu Publisher, Shanghai, 1984, p. 265.
4) J. Ji and Y. Zhao, "Shandong Yao Yong Dong Wu," Shandong Ke Xue Ji Shu Publisher, Jinan, 1979, p. 431.
5) G. L. Asherson and W. Dtak, *Immunology*, **15**, 405 (1968).
6) R. Kostar, M. Anderson and E. J. Dobber, *Fed. Proc.*, **18**, 418 (1958).
7) E. M. Glenn and B. J. Bowman, *Proc. Soc. Exp. Biol. Med.*, **130**, 1327 (1969).
8) C. A. Winter, E. A. Risley and G. W. Nuss, *Proc. Soc. Exp. Biol. Med.*, **111**, 544 (1962).
9) M. Yamazaki, Y. Noguchi, M. Tanda and S. Shintani, *J. Takeda Res. Lab.*, **40**, 163 (1981).
10) C. W. Dunnett, *J. Am. Statist. Assoc.*, **50**, 1096 (1955).
11) C. W. Dunnett, *Biometrics*, **20**, 482 (1964).
12) H. Scheffe, *Biometrika*, **40**, 87 (1953).