
SPECIES DIFFERENCE IN SUSCEPTIBILITY TO PHORBOL MYRISTATE ACETATE-INDUCED LEUKOPENIA AND LUNG INJURY: RAT VS. DOG

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Abstract—Phorbol myristate acetate (PMA) was administered intravenously in a single dose to rats (400 µg/kg) and in a single and repeated doses to dogs (40 µg/kg). Severe leukopenia was observed in both species. The leukopenia in rats was due to a decreased number of both lymphocytes and neutrophils while the leukopenia in dogs was mainly due to a decreased number of neutrophils. The rats recovered from leukopenia much faster than the dogs. The dog which received a single injection developed focal fibrosis in the lungs. Rats showed only slight localized hemorrhage in the lungs, although the rats received a ten-fold larger dose of PMA than the dogs. Lungs of dogs which received multiple injections revealed severe hemorrhagic lesions in most alveoli. Lung lesions induced by PMA are thought to be mediated by activated leukocytes. This suggests that the severity of lung lesion correlates with the degree and duration of neutropenia. In conclusion, intravenous administration of PMA caused lung damage in rats and dogs. However, rats show much less sensitivity to PMA than dogs, resulting from the different response of leukocytes to PMA.

Key words: Phorbol myristate acetate, leukopenia, lung injury, rat, dog, lymphopenia.

INTRODUCTION

PMA is well known as a potent stimulator of polymorphonuclear leukocytes (PMNL) (Repine et al., 1974; O'Flaherty et al., 1980a; DeChatelet et al., 1984),
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macrophages (Hoffman and Autor, 1982) and platelets (Zucker et al., 1974). In PMNL and macrophages, this agent activates the oxygen metabolic cycle resulting in the production of so-called active oxygen, i.e., superoxide anion, $O_2^-$ and $H_2O_2$. Recently it was reported that intravenous injection of PMA caused acute lung injury in various animal species: rabbits (O'Flaherty et al., 1980a; Shasby et al., 1982; McCall et al., 1983; Taylor et al., 1985), sheep (Loyd et al., 1983) and dogs (Mizer et al., 1989). These lung lesions are believed to be mediated by the active oxygen which is released from activated leukocytes (Johnson and Ward 1982; Shasby et al., 1982; Taylor et al., 1985; Allison et al., 1988). However, no one has succeeded in producing lung lesions in rats by intravenous administration of PMA, while intratracheal administration of PMA to rats (Johnson and Ward 1982; Kerr et al., 1987) and PMA exposure in perfused rat lung (Perry and Taylor 1988; Chou et al., 1990) caused acute lung injury. This leads us to investigate whether intravenous administration of PMA to rats can cause lung lesions through the activation of leukocytes as in other animal species. In the current study, we compare the pathologic and hematologic changes associated with PMA-induced lung injury in rats with those of dogs as a positive control.

**MATERIALS AND METHODS**

**Experimental animals**: Fifty-six male specific pathogen free Fischer rats (self supply), 6 weeks of age, weighing 100 to 140 g, and 4 male beagle dogs (self supply), 15 to 26 months of age, weighing 6.6 to 11.7 kg, were used. They were maintained in our Animal Care Facility and fed a standard laboratory diet with water provided *ad libitum*.

**Reagents**: A 2 mg/ml solution of PMA (Sigma) in dimethylsulfoxide (DMSO) was prepared and stored at $-20^\circ$C. Shortly before use, it was thawed and diluted to 400 $\mu$g/ml in saline. DMSO diluted to 0.2 ml/ml in saline (diluted DMSO solution) was used as a control.

**Experimental design**: Thirty-five rats were injected with a single injection of PMA (400 $\mu$g/kg of body weight) via the caudal vein. Five rats were necropsied periodically at each scheduled point 0, 1, 3, 5, 7, 12 and 24 hours after injection. As a control, 18 rats were injected with diluted DMSO solution (1 ml/kg). Three control rats were necropsied at each scheduled point in the same manner. Three dogs (Nos. 1, 2 and 3) were given either a single or multiple injections of PMA (40 $\mu$g/kg of body weight) via the jugular vein. As a control, one dog (No. 4) was given diluted DMSO solution (0.4 ml/kg) in the same manner. The experimental design in dogs is shown in Table 1. Dog No. 2 died 4 hours after the 3rd injection and dog No. 3 died 22 hours after the 4th injection, and both were necropsied immediately after death. Dog No. 1 was terminated by exsanguination under anesthesia and necropsied 10 days after a single injection. Dog No. 4 was not necropsied.
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**Hematological examination**: In rats, hematological examinations were performed on the blood collected at necropsy. In dogs, the blood was collected via the jugular vein and the examinations were performed periodically until animal death or termination of the experiment at 10 days after the 1st injection (Table 1). Erythrocyte (RBC) and total leukocyte (WBC) counts in peripheral blood were measured by a CC720 Counter (Toa, Japan). Neutrophil and lymphocyte counts were calculated from the differential cell counts of blood smears (Microx, Tateishi, Japan) and the total WBC counts.

**Table 1.** Experimental design in dogs.

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Note: a, blood was collected 0, 1, 2, 3, 4, 5, 7 and 12 hours after injection; b, collected 0, 1, 3 and 5 hours after injection; c, collected 0, 1 and 3 hours after injection; d, collected 0, 1, 3, 5, 12 and 24 hours after injection

**Pathological examination**: The lungs were removed from the rats and the dogs and 10% buffered formalin was instilled via the trachea to fill the periphery of the lungs. After fixation, the specimens were processed and embedded with paraffin in the usual manner. The paraffin sections were stained with hematoxylin and eosin (H and E), and examined with a light microscope.

**RESULTS**

**Hematological examination**: Rats: The RBC counts did not change throughout the experiment either in PMA treated (Fig. 1A) or control rats (data not shown). In contrast, WBC, neutrophil and lymphocyte counts varied drastically in the rats treated with PMA. One hour after
the PMA injection, all the white blood cell values sharply declined to the lowest levels: 16.3%, 18.2% and 16.4% of the values at time 0 in WBC, neutrophil and lymphocyte count, respectively. One hour after the injection, the numbers of neutrophils and lymphocytes began to increase and reached a plateau at 5 hours, then decreased again at 7 hours. Up to 7 hours after the injection, the WBC and lymphocyte counts were lower than the initial values measured at time 0; however, the neutrophil count increased over this time period to 308%, 429% and 279% of the initial values at 3, 5 and 7 hours post injection, respectively. Thereafter, the numbers began to increase to reach the highest level after 12 hours. After 12 hours, all WBC counts gradually decreased to approximately initial values (Fig. 1B). The WBC, neutrophil and lymphocyte counts in control rats showed little change throughout the experiment.
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Dogs: The RBC count was relatively stable in each of the three dogs treated with PMA throughout the experiment, with only a slight variation immediately after each PMA treatment (Fig. 2). On the other hand, both WBC and neutrophil counts decreased sharply after the single injection of PMA, i.e., 1 hour after injection the WBC count was 27.8%, 34.3% and 46.6%, and the neutrophil count was 23.7%, 23.7% and 61.7% of each initial value in dogs No. 1, 2 and 3, respectively. In all three dogs, 3 hours after the injection, the neutrophil and WBC counts began to increase and reached a plateau at 12 hours. After 12 hours those values again declined and showed the lowest value at day 1. In dog No. 1, after day 1, the WBC and neutrophil counts gradually increased and reached the highest level at day 3 (WBC count, $13.9 \times 10^3$/mm$^3$; neutrophil count, $9.5 \times 10^3$/mm$^3$). In dogs No. 2 and 3, each additional injection also caused an initial rapid reduction in WBC and neutrophil numbers. In dogs No. 2 and 3, the WBC and neutrophil counts measured just before additional PMA injections on days 4 and 3, respectively, were higher than on day 0. Lymphocyte counts in the three dogs also varied following each injection of PMA, although they were relatively stable compared to the WBC and neutrophil counts (Fig. 3). The control (dog No. 4), showed little change in WBC, neutrophil and lymphocyte counts (Fig. 4).

![Fig. 2. RBC counts of dogs injected with PMA. A, dog No. 1; B, dog No. 2; C, dog No. 3; P, PMA injection.](image-url)
Fig. 3. WBC counts and leukocyte differential counts in dogs injected with PMA. A, dog No. 1; B, dog No. 2; C, dog No. 3; P, PMA injection. Closed circle, solid line and dotted line show WBC, neutrophil and lymphocyte counts, respectively.

Pathological examination:

Rats: Alveolar damage characterized by slight hemorrhage was detected in a small area of each lobe at 1 hour after the PMA injection (Photo 1). The hemorrhagic changes were followed by a slight inflammatory reaction 3 to 12 hours after the injection. At 24 hours the hemorrhage declined and eosinophilic crystal was observed in some areas of lobes (Photo 2). No pathological changes were observed in control rats throughout the study.

Dogs: Hemorrhagic lesions were observed in all lobes of the lungs of dogs No. 2 and 3. The lesions consisted of alveolar wall disruption associated with formation of hyaline membrane, severe hemorrhage and inflammatory cell (neutrophil and macrophage) infiltration (Photo 3). Dog No. 1 had focal fibrosis in all lobes (Photo 4).
Fig. 4. RBC counts (A), WBC, and leukocyte differential counts (B) in control dog injected with DMSO. Closed circle, solid line and dotted line in Fig. 4B show WBC, neutrophil and lymphocyte counts, respectively. D, DMSO injection.
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Photo 1. Lung lesion from the rat which received a single injection of PMA (400 μg/kg), necropsied 1 hour after the injection. Slight hemorrhage is observed in some alveoli without disruption of alveolar wall. H and E, × 150.

Photo 2. Lung lesion from the rat which received a single injection of PMA (400 μg/kg), necropsied 24 hours after the injection. Eosinophilic crystal is present in some alveoli. Hemorrhage and cell infiltration are rarely seen. H and E, × 150.
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Photo 3. Lung lesion from dog No. 2 which received 40 μg/kg of PMA 3 times and died 4 hours after the 3rd injection. Severe alveolar wall disruption associated with formation of hyaline membrane, severe hemorrhage and inflammatory cell infiltration. H and E, × 120.

Photo 4. Lung lesion from dog No. 1 which received a single injection of PMA (40 μg/kg), necropsied at day 10. Focal fibrosis is observed. H and E, × 120.
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DISCUSSION

Hematological examination in the present study showed that PMA injection initially caused severe leukopenia in rats and dogs. In both species the WBC count in the peripheral blood decreased immediately after the PMA injection. It is well known that PMA stimulates PMNL to adhere, aggregate, degranulate and release oxygen radicals (Repine et al., 1974; O’Flaherty et al., 1980b; DeChatelet et al., 1984; Allison et al., 1988). This activation of neutrophils is considered to be the cause of leukopenia after PMA injection (O’Flaherty et al., 1980a; Shasby et al., 1982; Taylor et al., 1985; Mizer et al., 1989). In dogs the leukopenia was mainly due to neutropenia. On the other hand, both neutropenia and lymphopenia were observed in rats treated with PMA. Although PMA can stimulate lymphocyte proliferation or lymphokine production (Srendni et al., 1990), it has not been reported that PMA causes lymphopenia. Possibly activated macrophages or platelet aggregation in small capillaries after PMA injection (Zucker et al., 1974; Hoffman and Autor 1982) may affect the lymphocyte count in peripheral blood in rats whose main white blood cell component is the lymphocyte, or PMA may directly affect lymphocyte function to cause lymphopenia.

After leukopenia in dog No. 1 and in the rats which all received a single injection of PMA, the WBC and neutrophil counts in dog No. 1 and the rats, as well as the lymphocyte in the rats, increased. However, the recovery phase from the leukopenia was biphasic. Deactivation of activated leukocytes, recruitment of cells from the marginal pool or the hematopoietic system into the peripheral blood may increase the WBC count. Moreover, the inflammatory response to lung damage mediated by leukocytes and the turnover of leukocytes, whose circulating half-life is 5.5–7.5 hours (Latimer and Rakich 1989), could have important effects on the WBC count in peripheral blood. All of these factors might have contributed to the biphasic recovery from leukopenia.

In addition to the differing responses between rat and dog described above, the rats’ WBC count responded and returned to normal range much faster than the dogs’, although the rats received a ten-fold larger dose of PMA than the dogs. The respiratory burst in response to PMA has been documented in human (Opdahl et al., 1987), porcine (Makino et al., 1986), bovine (Morel et al., 1985), rabbit (Opdahl et al., 1987), and rat (Cooke and Hallett, 1985) neutrophils. The present study, however, suggests that the rat neutrophil has a lesser sensitivity to PMA than that of the dog. It is well known that there are marked species variations in neutrophil function (Styrt, 1989).

Pathological examination revealed that PMA caused lung lesions both in dogs and rats. These lesions, however, were especially severe in dogs. Taylor et al. (1985) reported that daily intravenous injections of PMA (40 μg/kg) in rabbits caused severe hemorrhagic pneumonitis followed by interstitial fibrosis. The dogs in this
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experiment showed similar pathologic changes.

In contrast, the rats developed only slight hemorrhagic alveolar damage which declined in 24 hours. These lesions are estimated to be much milder than those of dog No. 1 which received only a single injection. Johnson and Ward (1982) induced lung injury in rats with intratracheal administration of PMA. Considering the dose and the route of administration used in this experiment, this study suggests that the rat is much more resistant to PMA-induced lung injury than the dog and the rabbit (O’Flaherty et al., 1980a; Shasby et al., 1982; McCall et al., 1983; Taylor et al., 1985; Mizer et al., 1989).

As discussed above, PMA not only stimulates PMNL to adhere and aggregate but also activates the oxygen metabolite cycle of PMNL (Repine et al., 1974; O’Flaherty et al., 1980b; DeChatelet et al., 1984; Allison et al., 1988). The former action is considered to be the cause of leukopenia and the latter should be responsible for active oxygen production followed by lung injury. This study demonstrated that lung injury considered to be mediated by PMNL as well as leukopenia are milder in rats than in dogs. These results lead to the following conclusions: PMA caused hemorrhagic lung damage even in rats. However, rats show much less sensitivity to PMA than dogs, resulting from the different response of canine vs. rat leukocytes to PMA.

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REFERENCES


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