

Pigeon-serum-induced hypersensitivity pneumonitis in the dog

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Pigeon serum (PS) is one of the most common causes of hypersensitivity pneumonitis (HP). PS-induced HP was examined in a dog model. The dogs ($n = 6$) were immunized by i.m. injections of PS, followed by insufflation with aerosolized PS, while all control dogs ($n = 3$) received saline only. All animals insufflated with PS developed tachypnea 2–4 h after PS inhalation. After PS insufflation, a significant decrease in arterial oxygen tension (PaO_2) was detected in sensitized dogs. No change in PaO_2 was detected in sensitized dogs after saline or in the controls after PS insufflation. In intradermal skin tests with PS antigen, a positive skin reaction was found in 3/6 dogs in 30 min, and in 5/6 dogs in 6 and 48 h after the PS injections. Sensitized dogs showed a significant increase in PS-specific IgG in serum and lavage fluid (LF). In LF of sensitized dogs, an increase in the percentage of lymphocytes, eosinophils, and neutrophils was detected. Sensitized dogs developed chronic interstitial inflammation with lymphocytes, macrophages, plasma cells, and eosinophils in lungs. Granulomas with lymphocytes, histiocytes, and giant cells were detected in both the interstitium and the bronchiolar wall in the lungs of sensitized dogs. PaO_2 was lowest in dogs showing the most severe interstitial inflammation in the lungs. The results indicate that dogs can be successfully used in immunologic and physiologic studies of PS-induced HP.

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The clinical syndrome hypersensitivity pneumonitis (HP), also known as extrinsic allergic alveolitis, is caused by inhalation of organic materials (4, 15, 18). The disease is characterized by typical symptoms appearing 4–8 h after the exposure, presence of specific antibodies in patients' serum, granulomatous inflammation in the lungs, and restrictive ventilatory defect (4, 18).

Although a wide variety of causative agents have been associated with HP, by far the most common forms of HP are farmer's lung (12) and pigeon breeder's disease (3).

Although the causative agents and clinical characteristics of pigeon breeder's disease have been thoroughly investigated, the immunopathogenesis of the syndrome remains unresolved. It is evident that both antibody production and cell-mediated immunity are involved in the early stages of the disease (15). It is not clear, however, how the inflammation of the alveolar and interstitial areas of the lungs affects the diffusion of oxygen from the air space into the circulation, although we do know from clinical studies of HP patients that diffusion capacity and

arterial oxygen tension (PaO_2) are regularly decreased (4).

Several species of animals have been used to study pigeon-serum (PS)-induced HP to understand the pathogenic mechanisms of the disease (1, 5–7, 16). However, none of the previous studies have evaluated the dog model. Murine models are suitable for immunologic studies of HP (9, 19) but have limited use in lung function studies such as the assessment of diffusion capacity and ventilation/perfusion abnormalities. Therefore, a large-animal model was developed to use dogs to investigate the inflammatory and physiologic responses in lungs after exposure to PS. The results indicate that inflammation in the lungs included eosinophilic and neutrophilic granulocyte infiltration, granuloma formation, and thickening of alveolar septa.

Material and methods

Animals

Nine healthy purebred male beagle dogs weighing 13.1–16.4 kg were selected for the present study. Six

dogs were sensitized with PS, and three dogs served as controls.

PS antigen

PS was obtained from blood samples collected by cardiac puncture from anesthetized healthy pigeons. The blood samples were clotted and centrifuged, after which serum was collected and stored at -70°C until used. The PS samples used in the animal experiment were pathogen free.

Sensitization of the dogs

The dogs were sensitized first by one i.m. injection of 2.0 ml undiluted PS in four sites in each hind limb. Four weeks after the injections, insufflation exposure with PS was done three times a week for 4 weeks and once a month for 12 months with an ultrasonic nebulizer (DeVilbiss, Model 65, Somerset, PA, USA). In the insufflation exposure, the dogs inhaled the aerosolized PS through a tight mask for 15 min from the nebulizer, which contained 3.0 ml PS in 5 ml sterile saline. In all sensitized animals, specific antibodies against PS were detectable 4 weeks after the first injection of PS.

Physiologic procedure

Because we consider the degree of arterial hypoxemia to be a reliable index of the pathophysiologic effects of pneumonitis (4), arterial blood samples were taken from all dogs before and 3 h after insufflation with either PS or saline. Arterial blood was sampled in unanesthetized dogs by percutaneous catheterization of surgically exteriorized carotid arteries (10, 11). PaO_2 was measured with a calibrated blood gas analyzer (Radiometer ABL30, Copenhagen, Denmark) with correction for rectal temperature.

Skin testing

Before the skin tests, the fur was shaved from the skin area to be tested. Intradermal skin testing was done by injecting 100 μl 1:10 and 1:100 dilutions of PS in sterile saline both in PS-exposed dogs and in controls. Histamine dihydrochloride (1% solution) was used as a positive and sterile saline as a negative control. Skin test reactivity was observed 15–30 min, and 4–6, 24, 48, and 72 h after the injection of PS antigen. The skin reactions were graded from “–” to “+++” according to the diameter of the wheal reaction. The scale of the reaction was “+” if the diameter of the wheal in an immediate reaction and flare in the later skin reactions were 5–10 mm, “++” with 10–15 mm, “+++” with >15 mm, and “–” with no reaction.

Serum samples

Venous blood samples from the dogs were obtained before the sensitization, immediately before the last exposure, and when the dogs were killed. During the final operation, blood samples were obtained from anesthetized dogs by cardiac puncture.

Lung lavage and biopsy

Three hours after the last insufflation, the dogs were anesthetized with sodium pentobarbital (Nembutal, 30 mg/kg) and intubated with a 7.0-mm endotracheal tube. The chest was opened via midline sternotomy and the lungs were examined before the lung lavage and biopsies.

The left main bronchus was tightly closed and the lung lavage was done in the right bronchus. The lung biopsy was taken from the upper part of the lower lobe of the left lung. A sterile plastic cannula with outer diameter of 5 mm was introduced through the endotracheal tube into the right bronchus. A volume of 200 ml phosphate-buffered saline (PBS, pH 7.4) was injected in 50-ml aliquots through the cannula into the lungs. After each aliquot, the lavage fluid was immediately aspirated. The mean volume of fluid recovered was $75 \pm 8\%$ of the volume injected into the bronchus ($n = 9$). The volume of the lung lavage fluids (LF) was measured, filtered through a gauze, and centrifuged at 800 rpm for 5 min, and the supernatant was kept at -70°C for the assays of antibodies against PS antigens.

The sedimented cells of LF were washed three times in sterile PBS, and then the cells were suspended in PBS and prepared in a cytocentrifuge (Shandon Cytospin II, UK). Afterwards, microscopic slides were air-dried, fixed in methanol for 10 min, and stained with Wright's stain. The cell populations and biopsy specimens were examined with a light microscope by two investigators blind to the history of the exposure of the dogs.

Rabbit antisera against dog

Rabbit antisera against dog were obtained by s.c. injections of normal dog serum once a week for 6 weeks in rabbits. Blood samples were drawn 14 days after the last injection, and serum samples were stored in aliquots at -70°C and thawed for use as needed.

Enzyme immunoassay

The biotin-avidin-linked immunosorbent assay (BALISA) used in the present study has earlier been described in detail (8). Briefly, wells of polystyrene microtiter plates (Nunc-Immunoplate, Denmark) were coated with 100 μl PS antigen (1:5000 in PBS

Tween) by incubating for 3 h at 37°C and then overnight at 4°C. The wells were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBST). Any free binding sites left in the wells were blocked by treatment with 1% bovine serum albumin (BSA) in PBST for 1 h at 37°C. The wells were treated with 100 µl serum dilutions (1:1000) for 1 h at 37°C, and with rabbit antidog IgG (1:1000), biotinylated goat antirabbit IgG (1:1000, Sigma, St Louis, MO, USA), and streptavidin peroxidase (1:20 000, Sigma) each for 30 min at 37°C. The wells were washed three times with PBST between the addition of reagents. After washing, 200 µl chromogenic substrate (0.06% *o*-phenylene diamine and 0.015% H₂O₂ in citrate buffer, 0.1 M, pH 4.5) were added and incubated for 15 min. The reaction was stopped by addition of 20 µl H₂SO₄, and the adsorbance was measured at 490 nm with an ELISA reader (Mini-Reader, Dynatech, Chantilly, VA, USA). Appropriate controls were included in each plate.

Histologic studies

The excised, sterile lung biopsies were immediately transferred to the laboratory, and the tissue underwent inflation fixation by injecting buffered 4%

paraformaldehyde (1% glutaraldehyde solution under low pressure) through a syringe into a bronchiole or a blood vessel, and immersing the specimen covered with gauze in 10% formalin. After fixation of the lung biopsies, they were processed for paraffin embedding. The sections were then cut and stained with hematoxylin-eosin and hematoxylin-van Gieson's stains.

Statistical analysis

The means of the results in antibody measurements, LF cell counts, and arterial oxygen tension were compared by unpaired Student's *t*-test. A probability level of 0.05 or lower was regarded as statistically significant.

Results

All sensitized dogs which underwent insufflation of PS developed clinical symptoms varying from mild to severe. No animals died during the exposure period. Sensitized dogs insufflated with saline showed no symptoms. The most visible sign was tachypnea 2–4 h after PS inhalation. Control dogs showed no reaction after insufflation with PS.

Table 1. Summarized data of histologic changes and results of antibody measurements and skin tests in pigeon-serum (PS)-sensitized dogs

Finding	Sensitized dogs					
	1	2	3	4	5	6
<i>Histology*</i>						
Edema						
Interstitial	++	+	++	+	++	+
Alveolar	++	+	++	+	+++	+
Inflammation						
Interstitial	+++	+	+++	++	+++	+
Alveolar	++	+	++	++	+++	+
Bronchiolar	++	+++	+	++	+	+
Granulomas						
Parenchymal	+	-	+	-	++	-
Bronchiolar	+	+	-	-	+	-
Fibrosis						
Interstitial	+	+	-	++	+	-
Alveolar	+	-	-	-	+	-
Vascular changes						
Thick media	++	++	-	-	-	-
Vasculitis	-	+	-	-	-	-
<i>Antibodies against PS**</i>						
Serum antibodies						
OD value	0.347	0.378	0.318	0.240	0.304	0.251
Lavage antibodies						
OD value	0.389	0.365	0.432	0.430	0.344	0.355
<i>Skin test***</i>	-/+ / +++	++ / +++ / +++	- / + / +	+ / +++ / +++	+ / +++ / +++	- / - / -
ΔPaO_2	38.2	15.4	36.9	22.8	30.6	8.4

* Histologic changes in lungs of dogs (*n* = 6) sensitized with pigeon serum. Scale from - to +++ (- = not found, + = mild, ++ = moderate, and +++ = severe change).

** Serum and lung-lavage antibodies were measured with ELISA; results are expressed in OD values.

*** Intradermal skin tests used pigeon serum (1:10 and 1:100 dilutions).

Scale of reaction: - = no reaction, + = diameter of wheal and flare 5–10 mm, ++ = 10–15 mm, and +++ = >15 mm.

ΔPaO_2 = decrease in PaO_2 (mmHg) after PS challenge.

Physiologic findings

The PaO_2 values in the six experimental dogs are shown in Table 1 and Fig. 1. Three hours after insufflation of the sensitized dogs with PS, there was a significant decrease in PaO_2 ($P < 0.001$). When the sensitized dogs were insufflated with saline, there was no change in the PaO_2 . Control dogs insufflated with PS also showed no changes in PaO_2 .

Skin testing

No skin test reactions were observed in nonsensitized dogs insufflated only once with PS antigen or saline. Saline did not cause any skin test reactions in exposed dogs either. In all animals, histamine caused a skin reaction varying 3–5 mm in wheal diameter.

Three of six sensitized dogs showed immediate-, Arthus, and delayed-type skin reactions, while one dog did not show any skin reaction after PS injection (Fig. 2). Five dogs showed a flare reaction 4 and 48–72 h after PS antigen injection.

PS antibodies in dog serum and LF

The results of the antibody measurements in serum and LF appear in Fig. 3. The measurement of PS antibodies showed that all sensitized dogs produced

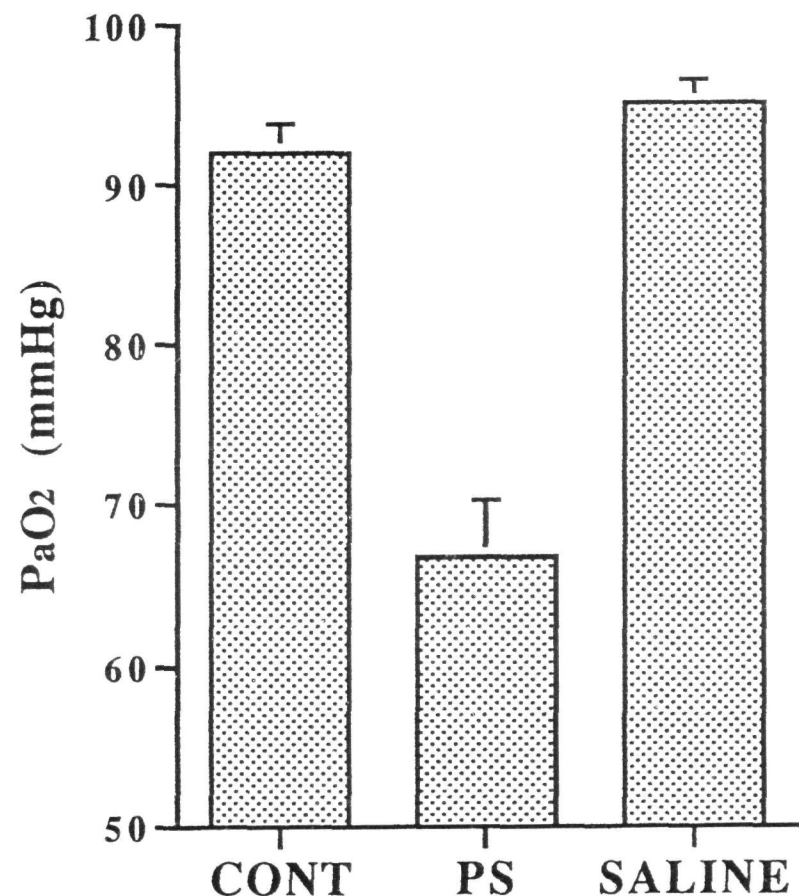


Fig. 1. Effect of pigeon serum and saline insufflation on mean arterial oxygen tension (PaO_2) in control (CONT) and PS-sensitized dogs.

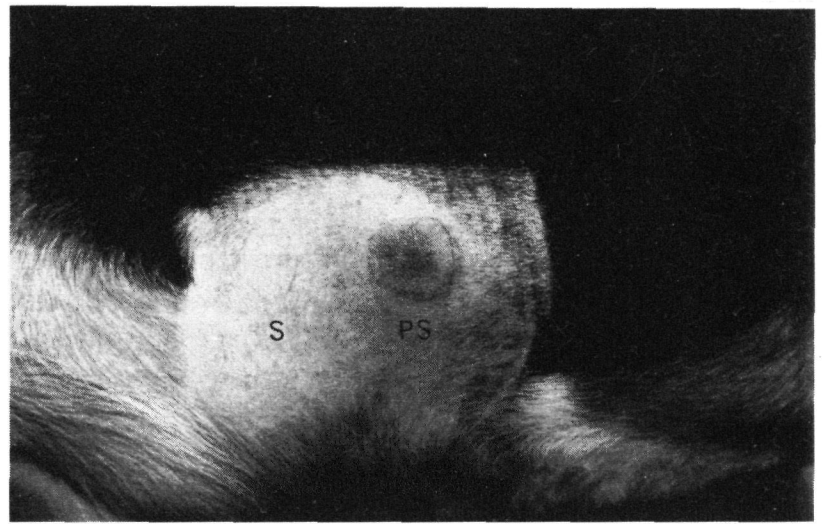


Fig. 2. Skin reaction in sensitized dog showing wheal and flare reaction 6 h after intradermal PS injection. S: saline.

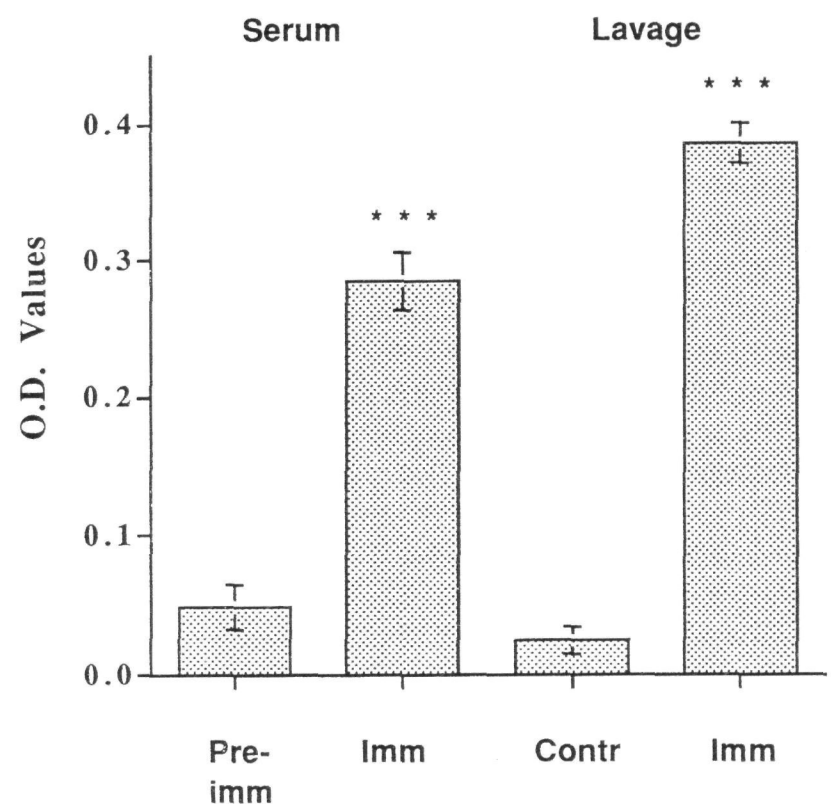


Fig. 3. Pigeon-serum-specific IgG antibodies in sera of immunized dogs ($n = 6$) before and after sensitization, and in lung lavage fluid of immunized dogs ($n = 6$) and controls ($n = 3$).

PS-specific IgG in serum (mean OD 0.31 ± 0.02). Before immunization, the six dogs and three controls also showed low levels of circulating IgG antibodies against PS. Their antibody levels, however, were significantly lower (mean OD 0.04 ± 0.01 ; $P < 0.01$) than in the dogs sensitized with i.m. injections and insufflation with PS. During the sensitization, the levels of circulating antibodies were elevated gradually, and the highest IgG levels against PS were detected 4 months after the first injection. After this, the levels remained unchanged.

For the measurement of PS-specific IgG in LF, lung lavage was done in six sensitized and three control dogs. The six dogs sensitized with PS showed specific antibodies against PS in LF (mean OD

0.39 ± 0.01) at levels significantly higher than those detected in controls (mean OD 0.02 ± 0.01 ; $P < 0.01$).

Lung lavage cells

The percentage of lymphocytes in sensitized dogs (mean 6%, range 4–9%) was significantly higher than in controls (0.5%, range 0.4–0.6%; $P < 0.01$), while the percentage of alveolar macrophages (mean 44%, range 37–87%) after insufflation was lower than in control dogs (mean 97%, range 95–99%; $P < 0.01$). In 4/6 exposed dogs (nos. 1–4 in Table 1), the percentage of polymorphonuclear granulocytes, especially neutrophils and eosinophils (mean 45%, range 35–50%), was elevated.

Histologic analysis

The histologic changes in exposed dogs are shown in Table 1. All PS-exposed animals developed chronic inflammation of the lungs, whereas lung specimens of nonexposed dogs did not show inflammation.

Alveolar and interstitial edema was detected in all exposed animals. In one dog, alveolar edema was severe. Interstitial inflammation with macrophages, lymphocytes, plasma cells, and eosinophilic and neutrophilic granulocytes was severe in three dogs (Fig. 4). Alveolar inflammation with macrophages, eosinophils, and neutrophils was at least moderate in four dogs. Bronchiolar inflammation with eosinophils, plasma cells, and macrophages was also detected. Parenchymal or bronchiolar granulomas with lymphocytes, histiocytes, plasma cells, and a few giant cells were present in four of six dogs (Fig. 5). In one dog, moderate fibrosis and in two dogs thickening of vascular wall were found.

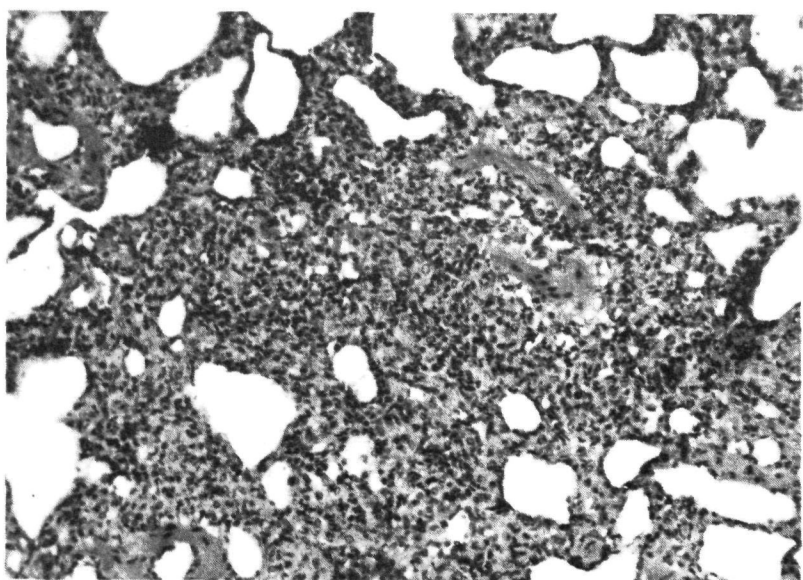


Fig. 4. Lung tissue of PS-sensitized dog showing interstitial inflammation with macrophages, lymphocytes, plasma cells, and eosinophilic and neutrophilic granulocytes (HE, $\times 100$).

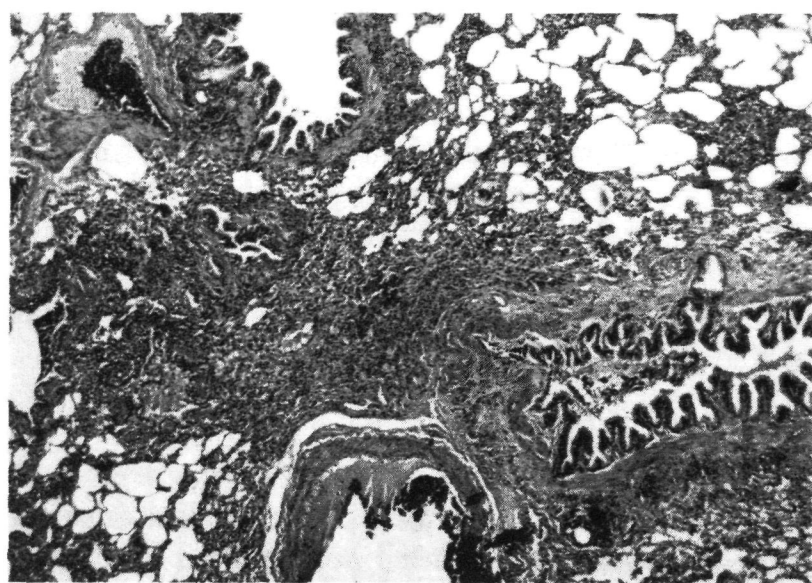


Fig. 5. Lung tissue specimen of PS-sensitized dog; bronchiolar granuloma with giant cell, lymphocytes, and plasma cells; thickening of alveolar septa; and interstitial inflammation are visible (HE, $\times 100$).

In two dogs which did not have granulomas in lung tissue, the PS-specific IgG levels were the lowest. In these dogs, which also had mild interstitial and alveolar inflammation, there was only a mild decrease in PaO_2 . The three dogs showing the most significant decrease in PaO_2 had severe interstitial inflammatory changes and at least moderate alveolar inflammation and edema. In two other dogs which did not show any fibrotic changes in the lung tissue, there was no immediate skin reaction.

Discussion

To our knowledge, this is the first description of PS-induced HP in dogs. The dog was chosen for the present study because of the large body of the animal for physiologic investigation, the ease of handling the animal unanesthetized, and the existence of already developed technology to study this species. Furthermore, when simultaneous observations of immunologic and physiologic changes after antigen challenge are needed, the canine model is superior to the murine because of the lack of physiologic methods for mice experiments. On the other hand, treadmill exercise in dogs can be used to investigate blood gas changes during exercise in diffuse interstitial lung disorders; this cannot be done properly in mice or primates. It is also remarkably easier to examine unanesthetized dogs than primates. Additionally, the canine model is less expensive than the primate.

To sensitize the dogs, we injected PS i.m. for 4 weeks before the insufflation of the PS antigen. After this, a significant increase in IgG antibodies against PS was detectable in the dogs' serum, and further insufflations with PS caused symptoms in sensitized animals.

HP is characterized by typical symptoms which appear 4–8 h after exposure to the antigen (4). In the

present experiment, all sensitized dogs developed fever, tachypnea, and difficulties in breathing. The peak of the symptoms was detected 3–4 h after the last exposure, at which time there was mild hypoxemia. The dogs recovered totally from the respiratory symptoms 8 h after insufflation.

As shown in the present PS-induced HP, symptomatic HP patients show specific antibodies. This feature has been used in the diagnosis of the disease (4). However, previous studies in PS-induced HP have shown that both symptomatic and asymptomatic subjects may develop detectable serum antibodies (4). Thus, the presence of PS-specific IgG antibodies either in serum or in LF is not pathognomonic of the disease and indicates only previous exposure to the relevant antigen (15).

In the present study, all sensitized animals showed a marked increase in the IgG antibodies against PS. The levels of PS-specific IgG were significantly higher in the serum samples collected from the sensitized dogs than in the preimmunized samples or the control sera. Low levels of PS antibodies were also detected in control serum samples. This may have been due to a previous exposure to PS antigen or to the cross-reactivity of PS antigen with other common inhalant allergens.

The sensitized dogs showed a significant increase in PS-specific antibodies in the lavage fluid. In the previous literature, there are only a few reports on the finding of specific antibodies in lavage fluid in man or animals. The lavage fluid antibodies may, however, play a major role in the cleavage of the offending antigen in the target organ, and the presence of antibodies in lavage fluid is clear evidence of a previous exposure.

Skin testing has been used in clinical diagnosis of PS-induced HP (4). The PS antigen has produced both immediate and delayed skin reactions in sensitized patients (4). In the present experiment, skin tests gave a positive reaction in 4/6 sensitized dogs 30 min after intradermal injection; in all of the skin-tested dogs, the test was positive 24 and 48 h after injection.

In LF, an increase in the number of lymphocytes was detected in all sensitized dogs. According to previous studies, an increase in lymphocytes, especially T cells, has been shown in HP and sarcoidosis (2, 14). In HP, however, the suppressor T cells are more numerous than T helper cells, while in sarcoidosis the ratio is reversed (2, 3). Interestingly, the number of neutrophilic granulocytes was increased 3 h after the final exposure, suggesting that these cells may play a major role in the acute state of the inflammatory process in the lungs before the accumulation of lymphocytes in the alveolar space. The finding of eosinophilia, which is not typical in patients with HP, suggests that these cells may have a

role in the immunopathogenesis of HP and may not be detected in patients' lavage because the LF is usually collected a few days after the last exposure. Furthermore, PS-induced immediate-type skin reactions also suggest the presence of IgE antibodies and eosinophils in sensitized subjects.

Histopathologically, allergic bronchioloalveolitis, with alveolar and interstitial edema, macrophages, granulomas, and lymphocytic and plasmocytic infiltrates, is the most typical finding in the lung tissue of HP patients (14, 18). Inflammatory changes are usually involved in the production of alveolar, interstitial, and bronchiolar granulomas (14, 18). Pulmonary tissue with granulomas and the presence of sensitized lymphocytes both in blood and LF strongly suggest that cell-mediated immunity plays a major role in the pathogenesis of HP (16, 17). Granuloma formation and antigenic material in lung tissue have earlier been detected in human HP (13, 18, 19). In accordance with the findings in human HP, granuloma formation with macrophages and lymphocytes was also detected in the peribronchiolar and intra-alveolar area in the present experiment. Interstitial and alveolar inflammation was present in all sensitized dogs. The mildest changes of the inflammation were associated with the smallest decrease in the PaO_2 , suggesting that the severity of the inflammation is related to the diffusion of oxygen from the alveoli to the capillaries.

Chronic changes occurred in four sensitized dogs in which fibrotic changes or intra-alveolar organization could be detected. Intra-alveolar organization has earlier been reported in human HP (18). In symptomatic patients, fibrotic changes usually indicate poor prognosis and irreversible damage of the lungs (18).

We conclude that the present animal model with PS-induced HP represents well the symptoms and the serologic and histologic findings seen in symptomatic HP patients. The acute histologic changes with interstitial and alveolar inflammation correlated with PaO_2 values. The canine model of HP may be especially useful for physiologic investigations in which the size of the animal is an advantage. The primary disadvantage is the lack of immunologic reagents available for dog experiments. The presence of eosinophils in lung tissue and positive immediate-type skin reactions after PS exposure in the present study suggest that IgE- and eosinophil-mediated immunomechanisms may play a significant role in PS-induced HP.

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