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Monitoring Delayed-Onset Pulmonary Hypersensitivity in Guinea Pigs

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Monitoring Delayed-Onset Pulmonary Hypersensitivity in Guinea Pigs. KAROL, M. H., STADLER, J., UNDERHILL, D. AND ALARIE, Y. (1981). *Toxicol. Appl. Pharmacol.* **61**, 277–285. To assess the total pulmonary sensitizing potency of an industrial chemical, consideration must be given to its ability to induce both immediate-onset and delayed-onset respiratory hypersensitivity reactions. An animal model system to evaluate immediate-onset responses has been described (Karol *et al.*, 1980). Measurement of delayed-onset sensitivity in animals may require continuous 48-hr monitoring of respirations. For this purpose a barometric chamber was employed for plethysmography. Delayed sensitivity was produced in guinea pigs by injection of Freund's complete adjuvant, a material classically used to induce delayed immunologic responses. Inhalation challenge of animals with purified tuberculin protein resulted in increased respiratory rate ($\bar{X}$ = 46% increase, $p < 0.001$) 9–12 hr following challenge. Respirations remained elevated for several hours before returning to preexposure levels. Control animals showed no increase. Histologic examination of pulmonary tissue revealed extensive mononuclear cell infiltration consistent with delayed hypersensitivity reactions. This system for measurement of delayed-onset responses has application for assessment of pulmonary sensitizing properties of industrial chemicals as well as testing the efficacy of drugs used for treatment of hypersensitivity reactions.

A number of industrial chemicals have been associated with delayed-onset respiratory hypersensitivity in workers. Examples include beryllium (Hardy and Tabershaw, 1946), formaldehyde (Hendrick and Lane, 1975), and toluene diisocyanate (Pepys *et al.*, 1972). Typically, symptoms develop several hours following exposure of sensitized individuals to the offending allergen. By contrast, responses occurring within 1 hr of exposure are designated "immediate-type" hypersensitivity. In order to evaluate the potency of chemicals as pulmonary sensitizers, it is necessary to consider both immediate- and delayed-onset reactions. We have previously reported development of an animal model for immediate-type respiratory tract hypersensitivity to inhaled chemicals (Karol *et al.*, 1980; Karol, 1980, 1981). In that model, guinea pigs were exposed to airborne chemicals on 5 consecutive days. Following a 2-week rest period, they were evaluated for pulmonary hypersensitivity by bronchial provocation challenge. Sensitivity was assessed by measurement of an increase in respiratory rate and decrease in tidal volume using animals restrained in body plethysmographs. While this procedure is useful for measurement of immediate-onset reactions, it is inappropriate for continued monitoring of animals to measure responses occurring after several hours. It was therefore necessary to investigate a system for recording changes in respiratory rate and tidal volume using unrestrained animals. This paper describes application of a rigid-

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walled barometric chamber for monitoring respiratory hypersensitivity reactions in unrestrained guinea pigs. Animals were sensitized to Freund's complete adjuvant and challenged with purified tuberculin protein, a system classically used to induce delayed-type immunologic response.

METHODS

Animals

Female English smooth-haired guinea pigs weighing 250–300 g (Hilltop Laboratories) were used throughout the study.

Sensitization

Twenty-eight guinea pigs were sensitized to Freund's complete adjuvant (FCA, Calbiochem) by injection of 50 μl of a saline–FCA emulsion (1:1, v/v) into each of the four foot pads. Control groups consisted of both noninjected animals (10) and sham-injected animals (4) which were inoculated with an emulsion of incomplete adjuvant (mineral oil)–0.15 M NaCl (1:1).

Skin Tests

Three to five weeks following sensitization, animals were tested for tuberculin hypersensitivity by intradermal injection of 50 μl purified tuberculin protein (PPD, Aplisol, 5 tuberculin units/0.1 ml, Parke-Davis) into shaved, depilated dorsal sites. Sites were evaluated using a scale of 0 to +++ for redness, swelling, and induration. A reaction of 3 mm received a grade of +.

Inhalation Challenge

Pulmonary reaction was assessed by bronchial provocation challenge with PPD. Equal volumes of PPD (100,000 tuberculin units/ml, Connaught Laboratories Limited, Ontario, Canada) and water were mixed, and placed in an all-glass Dautrebande generator operated at 1 kg/cm^2. For challenge, animals were placed in individual body plethysmographs which were connected to a 10-liter Plexiglas chamber (Karol *et al.*, 1978). Heads of animals extended into a chamber though latex dams. Air was drawn through the chamber at a rate of 20 liters/min. The concentration of PPD in the chamber was 24 μg/liter air by sampling onto a 0.8 μm Millipore filter. Particle size was determined using an Anderson mini-impactor. Particles had a mass median diameter of 2.6 μm and a count median diameter of 0.38 μm (geometric standard deviation of 2.28). To establish specificity of pulmonary response, four FCA-sensitized animals were challenged with an aerosol of an immunologically unrelated protein, ovalbumin. These challenges were conducted using aerosol concentrations and conditions as described for inhalation challenge with PPD. Respiratory rates of animals were monitored during inhalation challenge using a pressure transducer attached to the body plethysmograph (Karol *et al.*, 1978). Respiratory rate measurement during the 48 hr following challenge was accomplished using the system described below.

Measurement of Delayed Pulmonary Reaction

Physiologic measurement of response. A variety of pathologic conditions stimulate pulmonary irritant, stretch, or type J receptors (Alarie, 1973; Widdicombe, 1974, 1977). Examples of such conditions include pulmonary congestion and interstitial edema. Receptors are also stimulated by mediators released during respiratory anaphylaxis. The stimulation results in a reflex increase in respiratory rate (Alarie, 1973; Widdicombe, 1974, 1977). This response has been noted in a number of animal species during immediate as well as delayed pulmonary hypersensitivity reactions (Alarie, 1981). In guinea pigs, the degree of respiratory rate increase has been used to assess the severity of immediate hypersensitivity reactions (Karol *et al.*, 1978, 1980).

Plethysmography for measurement of delayed-onset responses. In 1868, Bert (1868) observed that when an animal is placed in a rigid-walled chamber, i.e., a whole body plethysmograph, a change in pressure can be recorded with each breath. The pressure change in the chamber (ΔP_c) is caused by warming and humidification of air drawn into the lung from the chamber during inspiration. The reverse occurs during expiration (Chapin, 1954). This occurrence has been noted by numerous researchers, most recently by Jacky (1978) and Epstein *et al.* (1980).

In the current study the rigid-walled chamber was adapted for use in measuring respiratory rates of guinea pigs. As schematically presented in Fig. 1, the animal was placed in a hermetically sealed clear plastic chamber for 48 hr. Sufficient food was provided for this period and bedding was included to absorb liquid wastes. The volume of air in the chamber when bedding and animal were present was approximately 2 liters. Airflow through the chamber was regulated at 2 liters/min thereby providing 12 air changes per hour (Silver, 1946). This airflow assured no accumulation of ammonia (Barrow and Dodd, 1979) or carbon dioxide and no depletion of oxygen. The temperature rise with the animal in the whole body plethysmograph was less than 0.5°C.

A long tube was used for the inlet of the chamber. The tube served as a resistance to effectively "seal" the plethysmograph. In this manner ΔP_c could be recorded without appreciable loss (Fig. 2). This was verified by

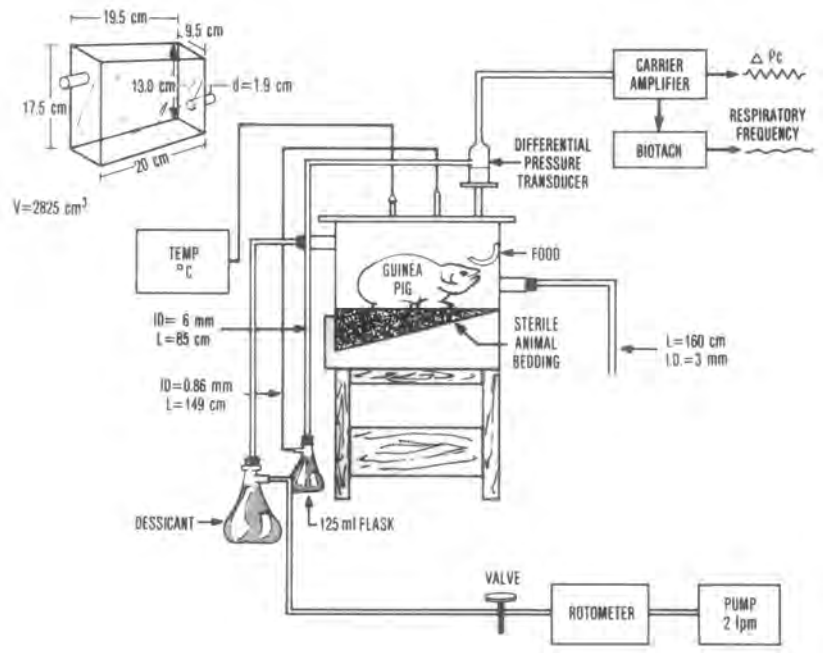

FIG. 1. Plethysmograph for long-term measurement of respiratory rate.

recording ΔP_c with flow through the system as compared with both inlet and outlet closed during quiet breathing of an animal. A Statham PM 197 differential pressure transducer was used for measurement of ΔP_c. One port of the transducer was connected directly to the chamber while the other port was connected to the chamber via a flask and a long tube of small diameter to create a resistance-capacitance (RC) system having a time constant of approximately 10 sec. This was required to compensate for the static negative pressure created in the chamber by the constant airflow (i.e., 2 liters/min) and to prevent baseline drift of ΔP_c due to changes in flow across the inlet tube. The signal from the pressure transducer was displayed on a Gould 200 oscillograph (Fig. 2) and also fed into a Gould Biotachometer set in the averaging mode. The ouput of the Biotachometer was displayed on a recorder at a paper speed of 20 cm/hr to monitor the average breathing frequency of each animal (see Fig. 3).

During pulmonary hypersensitivity reactions, tidal volume (VT) typically decreases. In this study, VT decrease was apparent on oscillograph tracings of challenged guinea pigs. However, tracings were not calibrated to yield absolute values for tidal volume.

Acclimatization of Animals to the Barometric Chamber

Immediately following inhalation challenge, animals were transferred from the exposure chamber to the barometric chamber for a 48-hr continuous measurement of respiratory rate. Placed in the new surroundings animals typically explored all areas and openings for 1–2 hr. This movement created difficulty distinguishing respiratory rate from other movements as illustrated in Fig. 3. Respiratory rates of nonacclimatized animals during the first 2 hr following challenge were usually difficult to assess (Fig. 3A). By 2 hr, animals became less active and respiratory rates were discernible.

The frequent loss of respiratory rate data during the first 2 hr following challenge prompted attempts to acclimatize animals to the chamber. Placing animals in the chamber for 1 or 2 hr prior to challenge was not sufficient to prevent excessive movement, but housing guinea pigs overnight in the barometric chambers proved effective in reducing the amount of exploratory movement when guinea pigs were returned to the chambers following inhalation challenge. Respiratory rate tracings from acclimatized and nonacclimatized animals are compared in Fig. 3. Without acclimatization, excessive movement obscured respiratory rate data for 1.5–2 hr. After 2 hr in the chamber, the actual breathing rate of the animal was apparent. By contrast, acclimatized animals usually settled within 40 min and accurate rate data could be obtained within the first hour following challenge (Fig. 3B).

Histologic Evaluation of Pulmonary Tissue

Sensitized animals and controls were sacrificed by injection of an overdose of pentobarbital at 10, 24, or

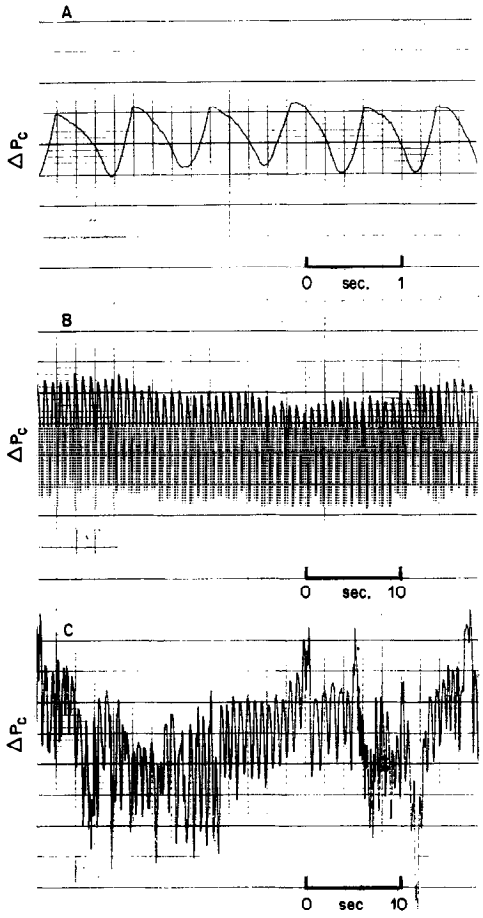

FIG. 2. Changes in pressure in the chamber, ΔP_c. Tracings A and B: during quiet breathing, using different oscillograph recording speeds. Tracing C: Typical changes in baseline due to chewing and body movement of the animal interfering with continuous recording of respiratory rate.

48 hr following inhalation challenge. Lungs were removed and inflated with 10% buffered formalin. Sections of trachea, bronchial airways, and pleural areas were prepared and stained with hematoxylin and eosin. Sections from the midplane and pleural areas showed greatest mononuclear cell infiltration in experimental animals and were used for evaluation of reactions.

Statistical Evaluation

Data were assessed for statistical significance by Student's *t* test.

RESULTS

Dermal Sensitivity

Skin tests were employed to identify those guinea pigs, injected with Freund's complete adjuvant, having possible immunologic sensitivity to tuberculin protein. Positive reactions were noted in 11 of 28 experimental animals with reactions developing 18–24 hr following intradermal challenge. Animals with severe skin reactions were selected for inhalation challenge.

Respiratory Sensitivity

Results of bronchial provocation challenge of six FCA-sensitized guinea pigs, with and without prior acclimatization, are given in Table 1. Each animal responded to PPD inhalation challenge with an increase in respiratory rate. Maximal values occurred 9–12 hr following challenge. Increases were detected regardless of whether or not animals had been acclimatized to the chamber (see for example guinea pigs 1, 2, and 3).

Respiratory responses of four sets of control animals are summarized in Table 2. The first group was composed of seven animals which were neither injected for sensitization

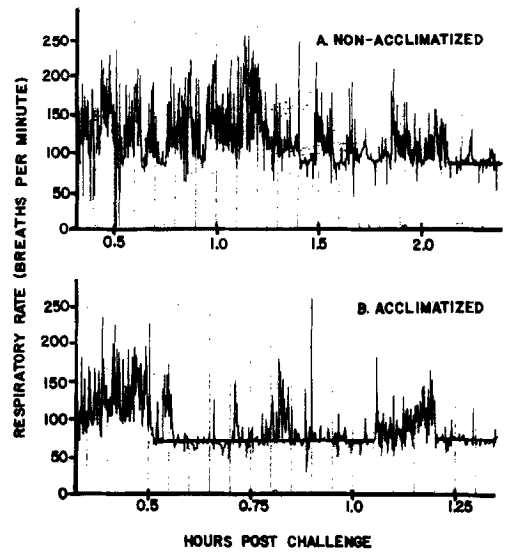

FIG. 3. Continuous recording of respiratory rates of guinea pigs following inhalation challenge. Solid lines represent the respiratory rate at the times indicated. (A) Nonacclimatized animal, excessive movement obscured breathing rate tracing until 2.25 hr postchallenge. (B) Acclimatized animal, breathing rate discernible 0.5 hr postchallenge.

TABLE 1

DELAYED-ONSET RESPIRATORY RESPONSES OF FCA-SENSITIZED GUINEA PIGS TO INHALATION CHALLENGE WITH PPD

Animal No.[a]	Respiratory rate		Percentage increase	Lung histology[d]
	Normal[b]	Maximum[c]		
1	83	120 (12-13)	45	N.D.[e]
1	80	120 (12-13)	50	N.D.
2	75	104 (10-11)	39	N.D.
2[f]	72	108 (9-10)	50	Pos. (10 hr)
3	77	107 (12-13)	39	N.D.
3[f]	75	103 (9-10)	37	Pos. (24 hr)
4[f]	70	121 (9-10)	73	Pos. (48 hr)
5[f]	82	109 (9-10)	32	Pos. (48 hr)
6[f]	77	115 (11-12)	49	Pos. (48 hr)
$\bar{X}$ ± SD	77 ± 4	112 ± 7	46 ± 12	

[a] Animals 1, 2, 3 each received two bronchial provocation challenges spaced 14 days apart.

[b] For acclimatized animals the normal respiratory rate was calculated from measurements obtained during the first hour following challenge. For nonacclimatized animals, measurements at 30 hr following challenge were used (see Fig. 4).

[c] Maximum respiratory rate was the highest rate following inhalation challenge calculated from the average of four consecutive readings made at 15-min intervals. The time of appearance (in hr) of the maximum rate is indicated in parentheses.

[d] Positive sections were characterized by extensive mononuclear cell infiltration into alveolar spaces and thickening of alveolar walls. Lungs were obtained at the indicated hour following inhalation challenge.

[e] Histologic examination was not performed.

[f] Animals were acclimatized to the chamber prior to challenge.

nor challenged. Respirations of these animals were monitored continuously for 48 hr to determine possible circadian patterns in respiratory rate. The mean percentage change in respiratory rate during 48 hr in control Group I was 2 ± 12 (mean ± SD). This value was significantly different ($p < 0.001$) from the mean increase of 46 ± 12 noted with FCA-sensitized, PPD-challenged guinea pigs. Respiratory rate increases found in control Groups II, III, and IV were −3 ± 5, 5 ± 13, and 5 ± 3, respectively. Compared with each of these groups, the mean respiratory rate increase of experimental animals was highly significant ($p < 0.001$).

Control Group IV was established to examine the specificity of the delayed-onset pulmonary responses. This group consisted of four FCA-injected guinea pigs, each of which demonstrated substantial skin test sensitivity to PPD. Bronchial provocation challenge of these animals with the antigenically unrelated protein, ovalbumin, produced no significant change in respiratory rate (Table 2) during the 48 hr following inhalation challenge.

TABLE 2

RESPIRATORY MEASUREMENTS OF CONTROL GROUPS OF GUINEA PIGS

Animal No.	Respiratory rate		Percentage increase
	Normal	Maximum[a]	
Group I: Noninjected, no inhalation challenge			
11[b]	80	81 (4-5 hr)	1
12[b]	74	93 (8-9 hr)	25
13[b]	70	74 (7-8 hr)	6
14[b]	81	73 (10-11 hr)	−10
15[b]	74	72 (7-8 hr)	−3
16[b]	65	61 (5-6 hr)	−6
17[b]	60	59 (12-13 hr)	−2
$\bar{X}$ ± SD	72 ± 8	73 ± 12	2 ± 12
Group II: Noninjected, PPD inhalation challenge			
12[b]	90	85 (17-18 hr)	−6
13[b]	77	80 (2-3 hr)	4
14[b]	50	46 (7-8 hr)	−8
15[b]	60	61 (2-3 hr)	2
16[b]	56	55 (12-13 hr)	−2
17[b]	57	53 (2-3 hr)	−7
$\bar{X}$ ± SD	65 ± 12	63 ± 16	−3 ± 5
Group III: Injected with incomplete adjuvant, PPD inhalation challenge			
11[b]	74	68 (2-3 hr)	−8
18[b]	75	80 (2-3 hr)	+7
19	76	89 (3-4 hr)	17
$\bar{X}$ ± SD	75 ± 1	79 ± 11	5 ± 13
Group IV: Injected with FCA, ovalbumin inhalation challenge			
23[b]	67	73 (2-3 hr)	9
24[b]	77	78 (2-3 hr)	1
25[b]	63	67 (4-5 hr)	6
26[b]	60	63 (3-4 hr)	5
$\bar{X}$ ± SD	67 ± 7	70 ± 7	5 ± 3

[a] Calculated as described in Table 1. The time of appearance (in hr) of the maximum rate is shown in parentheses.

[b] Animals were acclimatized to the chamber prior to challenge.

Respiratory rates of experimental and control animals were monitored for 48 hr following inhalation challenge. Results obtained with three sensitized and three control guinea pigs are shown in Fig. 4. In FCA-injected animals, respiratory rates typically started to increase by 6 hr postchallenge and maximum responses were seen at 9–12 hr. Thereafter respiratory rates gradually decreased and returned to normal preexposure values approximately 30 hr postchallenge. By contrast, no change in breathing rate was noted in control animals during the entire 40-hr period following challenge.

Histologic Evaluation

Lung sections obtained from FCA-injected animals at 10, 24, or 48 hr following inhalation challenge consistently showed mononuclear cell infiltration (see Fig. 5A). The most extensive changes were in specimens taken 48 hr postchallenge. Sections were characterized by infiltration of lymphocytes and thickened alveolar walls. Macrophages were frequently observed in alveolar spaces and clusters of mononuclear cells occurred at alveolar junctions. No capillary involvement was seen. Lung sections from control animals (Groups I–IV) were obtained 48 hr following challenge and examined for cell infiltration (Fig. 5B). These sections showed no evidence of cellular infiltration or thickening of alveolar walls.

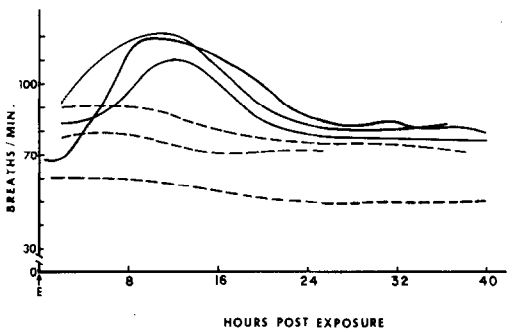

FIG. 4. Respiratory rates of three sensitized (solid lines) and three control (broken lines) guinea pigs monitored continuously for 40 hr following inhalation challenge with PPD aerosol.

DISCUSSION

Miyamoto *et al.* (1971) examined respiratory function in guinea pigs sensitized to Freund's complete adjuvant. From intermittent measurement of respiratory rate, these authors detected an increase in respiratory frequency beginning 6 hr after inhalation challenge and reaching maximum values in 24–48 hr. They could not detect a significant change in airway resistance during this period. Histologic studies supported delayed-type hypersensitivity. Wilkie *et al.* (1980) reported elevation of respiratory rates in sensitized calves several days following inhalation challenge. In the present study, both physiologic and pathologic measurements were used to assess delayed pulmonary hypersensitivity.

The delayed-onset respiratory reaction was characterized by increased respiratory frequency beginning several hours following inhalation challenge with antigen. In order to detect the response, which has an unpredictable appearance time, use was made of a barometric chamber originally described by Bert (1868). Employing airflow through the chamber of 2 liters/min, it was possible to monitor respirations of animals continuously for 48 hr.

In order to obtain reliable values for respirations during the first 2 hr following challenge when the guinea pig was placed in the barometric chamber, it was necessary to condition animals to the chamber. This acclimatization was accomplished most conveniently by housing animals in the chamber during the evening prior to a morning inhalation challenge. Failure to familiarize the animal with the chamber in this manner resulted in extensive exploration of the chamber and, as a consequence, interference in detecting respiration-related pressure changes in the chamber.

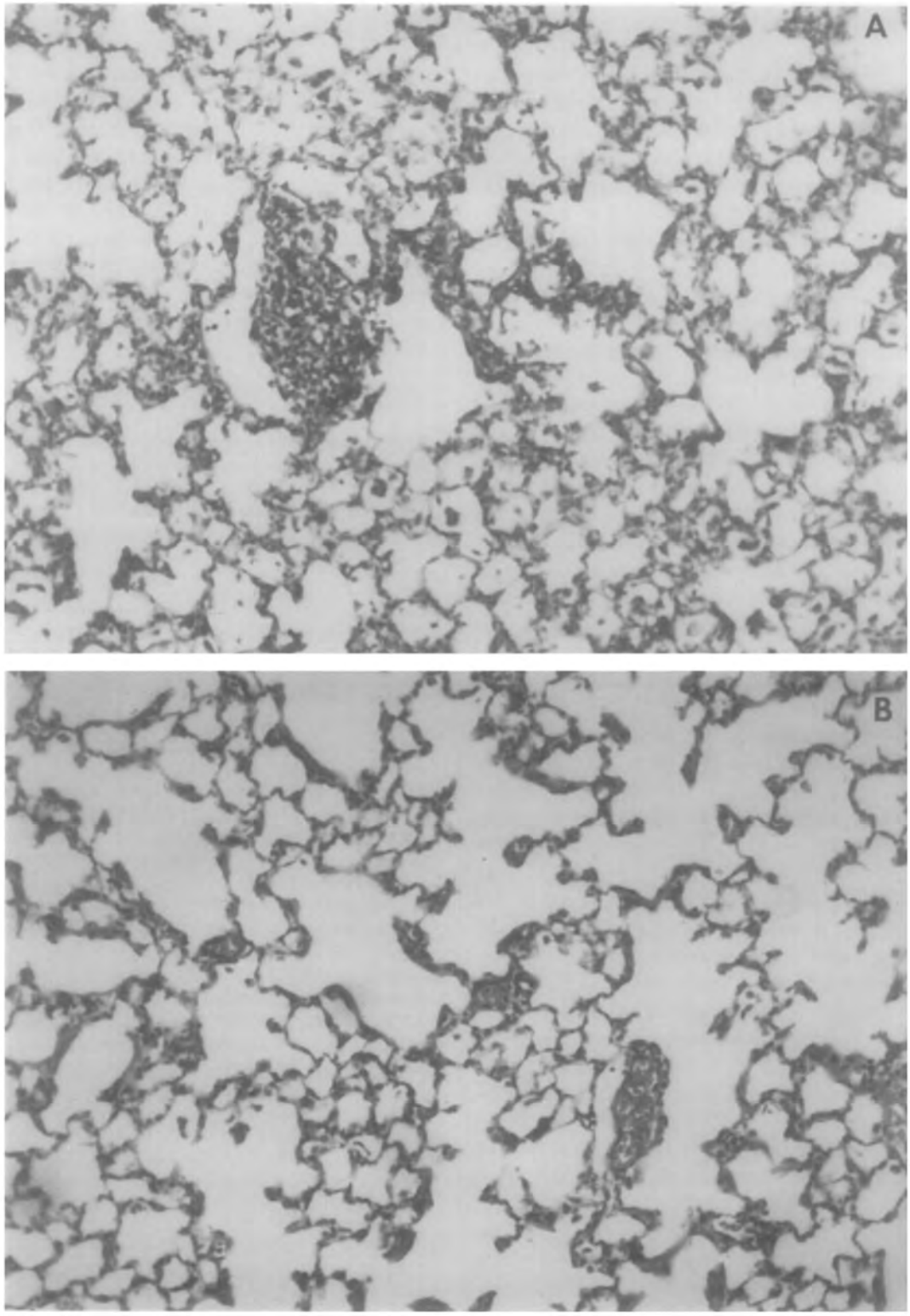

FIG. 5. Histologic appearance of lungs of a sensitized (A) and control (B) guinea pig 48 hr following inhalation challenge with PPD aerosol.

The delayed-onset pulmonary response differed in several respects from the immediate-onset pulmonary reactions studied previously using guinea pigs sensitized by inhalation exposure to proteins, hapten–protein conjugates, or haptens (Karol *et al.*, 1978, 1980, 1981). The immediate respiratory reactions were characterized by respiratory rate increases of 50 to 150% and typically lasted for 3 to 10 min before returning to normal prechallenge values. In the study reported here respiratory rate increases ranging from 32 to 73% were observed 9 to 12 hr following inhalation challenge and lasted several hours. A difference between immediate and delayed-onset reactions also was apparent from histologic examination of lung sections. Lungs taken at the time of immediate-onset reactions or 48 hr later showed no abnormal features. By contrast, sections of lungs taken at the time of delayed-onset responses (10 hr postchallenge) showed mononuclear cell infiltration and alveolar wall thickening typical of delayed immune responses. These features were not detected in any of the control animals sacrificed at 10, 24, or 48 hr following challenge with PPD aerosol or in experimental animals following aerosol challenge with an unrelated antigen, ovalbumin.

The pulmonary reaction observed in the current study had several characteristics suggestive of classical immunologic cell-mediated delayed hypersensitivity, i.e., a 9- to 12-hr delay before maximum response and histologic demonstration of mononuclear cell infiltration into the lung. In addition, skin tests indicated the presence of delayed dermal hypersensitivity to PPD antigen. However before the pulmonary response can be equated with classical cell-mediated delayed hypersensitivity, further experiments are needed. Passive transfer of the response with serum or lymphoid cells would be appropriate.

In conclusion, use of a barometric chamber allowed continual monitoring of animals for respiratory frequency. Using this chamber, delayed-onset pulmonary reactions were detected 6 to 10 hr following inhalation challenge and persisted for several hours. The plethysmograph should be of value for study of many pulmonary disorders in which continued measurement of respiratory frequency is important. It also possesses potential for use in evaluating drugs for effective treatment of adverse delayed-onset pulmonary hypersensitivity reactions.

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