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Anti-Inflammatory Assay. An Improved Method Based on the Reversal of Endotoxin-Induced Lung Inflammation in Mice. (29490)

A. MELI, C. R. SMITH AND A. WOLFF (Introduced by W. M. Govier)
Department of Physiology, Warner-Lambert Research Institute, Morris Plains, N. J.

Inhalation of aerosolized bacterial endotoxin (S. Abortus Equi) induces in mice severe inflammation and increase in weight of the lung. The observation that these effects could be reduced by subsequent administration of anti-inflammatory agents led to the development of an anti-inflammatory assay (1).

In its original design, however, the aerosolization chamber presented some inconveniences: (a) tendency of the animals to crowd in a limited area of the chamber; (b) occasional deposition of feces in the open end of the nebulizer, resulting in occlusion of the nozzle, which required repetition of the test; (c) use of vaseline as a seal between the dome and the stand, and (d) the limited number of animals per each exposure.

With the apparatus described here, not only were these inconveniences eliminated, but it was observed that a concentration of endotoxin equal to that used previously(1) could produce a comparable degree of inflammation and increase in lung weight when administered to a double number of animals. This observation prompted us to investigate the effect of lower concentrations of endotoxin.

Materials and methods. The apparatus is shown in Fig. 1 and 2. The Black, Sivalls and Bryson regulator with pressure gauge, is attached directly to the pressurized air outlet. The DeVilbiss No. 40 nebulizer is connected to the regulator by Tygon tubing (18″ long, ¼″ i.d.) and attached to the stand *via* a ⅞″ rubber stopper with a ⅝″ hole in the center.

The plastic dome is 9″ high with an inner diameter of 10¼″. The outside diameter at the base is 11½″. The upper part of the dome begins to assume its concave appearance approximately 4½″ from the base. A rubber "half round" gasket (¼″ wide, ⅛″ thick) is glued to the peripheral edge of the base of the dome.

The round cage in which the animals stay during aerosolization consists of: (a) a 5/16″ square wire mesh platform (10″ i.d.) with a 3¼″ hole in the center, (b) 2 concentric walls, 4¼″ high, 10″ and 3¼″ i.d., respectively, made of galvanized sheet metal (27 gauge) and (c) a 5/16″ square wire mesh cover with a 3¼″ hole in the center, kept in place by 3 galvanized tabs bent at a 90° angle and soldered to the periphery of the cover. The inner part of the cage (Fig. 2) is divided into 4 sections, each one large enough to house 5 mice. The cage is sup-

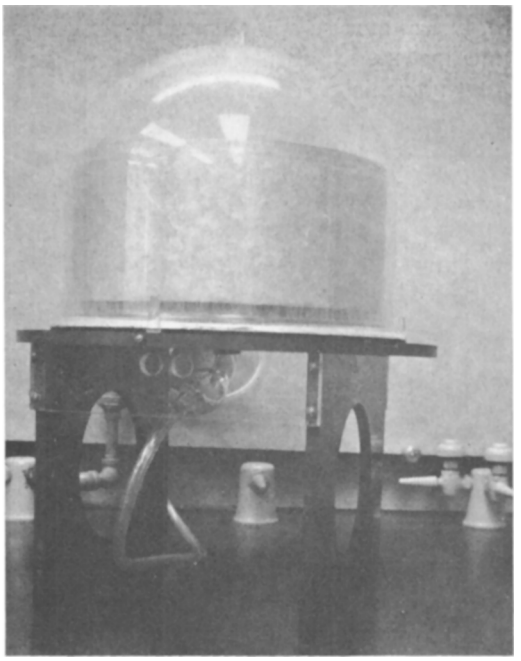

FIG. 1. Apparatus.

ported 7/8" above the stand by means of 3 stainless steel bolts.

The cage and the dome are mounted on a Bakelite disc (13½" i.d., 3/8" thick) with a 7/8" hole in the center. Three Lucite dowels (3/8" i.d., 1½" long, tapered to 3/16" i.d.) are placed into drilled depressions of the disc, equidistant from each other and 6" from the center. The purpose of these dowels is to confine the dome and the cage to a specific area of the stand.

The stand is composed of 5 elements: the Bakelite disc, 2 Bakelite vertical supports (9 3/16" × 8" × 3/8") and 2 Lucite brackets (2" × 8¾" × 3/8"). The vertical supports have a 5" hole in the center to facilitate the manipulation of the nebulizer. The 2 Lucite brackets are attached to the vertical supports immediately below the disc.

One sheet of filter paper (11½" i.d.) with a 7/8" hole in the center, is placed on the Bakelite disc to receive the waste products of the animals.

Groups of 20 Charles River CD-1 (pathogen-free) female albino mice, weighing 18-20 g, are equally distributed among the 4 sections of the cage. The cage is then placed on the stand, covered by the dome and the air turned on to a preset pressure of 25 PSI. As a safety precaution the apparatus is placed in a hood with the exhaust fan in operation. Each group of mice (with the exception of the untreated control group) receives exactly 4 ml of aerosolized aqueous endotoxin solution. The most consistent results were obtained when using a DeVilbiss No. 40 nebulizer which delivers this amount in approximately 4-5 minutes. The concentrations of endotoxin (S. Abortus Equi—Difco Labs.) used in these experiments were: 12-9-3-1-0.33 and 0.11 mg/ml. Endotoxin treatment was repeated 24 hours later and the animals randomized after each exposure.

The animals were killed by CO_2 asphyxiation 48 hours after the second exposure to endotoxin. The lungs of the animals exposed to the various concentration of endotoxin were carefully excised and after the lung weight was recorded, placed in 1% neutral formalin. After the tissue was adequately fixed, cross sections of 3-5 mm thickness were taken from each lobe of the lung. The sections were subsequently dehydrated in alcohol, cleared in toluene and infiltrated with Paraplast. The sections were cut at 4 mμ, stained with hematoxylin-eosin and examined.

For reasons which will become obvious after the results are considered, the 3 mg/ml concentration of endotoxin was chosen to determine the anti-inflammatory activity of cortisone acetate, aspirin and phenylbutazone. These compounds were administered as aqueous suspensions,* 6 and 24 hours after the second exposure to endotoxin. A

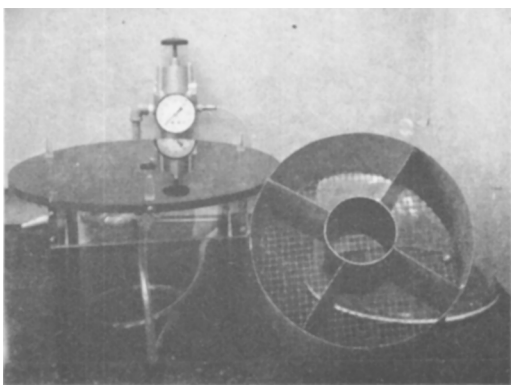

FIG. 2. Apparatus.

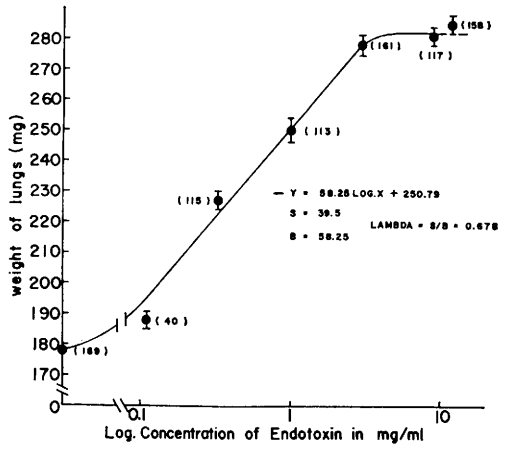

FIG. 3. Effect of graded doses of endotoxin upon lung weight in mice. The numbers in parentheses indicate the total number of observations.

one percent Tween 80 solution was used as a suspending vehicle. The animals were killed by CO_2 asphyxiation 24 hours after the second administration of the anti-inflammatory compounds. The anti-inflammatory activity is expressed as percent reversal of the increase in lung weight and was determined according to the formula:

$$\frac{\text{Endotoxin treated} - \text{Endotoxin and compound treated}}{\text{Endotoxin treated} - \text{Untreated control}} \times 100.$$

Results. The results obtained after exposure of the animals to the various concentrations of endotoxin are reported in Fig. 3. The increase in lung weight was proportional to the concentration of endotoxin used up to 3 mg/ml. Above this concentration no further significant increase in weight of the lung was observed.

Histological examination of the lungs of the animals exposed to the various concentrations of endotoxin (Fig. 4) gave the following results:

1) At the 0.11 mg/ml concentration, the lungs are comparable to the controls.

2) At the 0.33 mg/ml concentration, the bronchial mucosa and the pulmonary parenchyma are slightly edematous. There is a minimal amount of inflammatory infiltration.

3) At the 1 mg/ml concentration, the bronchial mucosa and pulmonary parenchyma are moderately edematous. The lumens of the bronchioles contain exudate in which some polymorphonuclear elements and round cells are noted.

4) At the 3 mg/ml concentration, the bronchial mucosa is markedly edematous. The pulmonary parenchyma presents a moderate infiltration of inflammatory cells. The lumens of the bronchioles contain exudate in which polymorphonuclear elements and round cells are noted. Some of the alveolar spaces are filled with fibrinous exudate.

5) At the 12 mg/ml concentration, the histological picture is similar to that described above.

The data in Table I show that cortisone acetate and Phenylbutazone exhibited similar and significant anti-inflammatory activity at both dose levels. The high dose of aspirin produced a comparable degree of anti-inflammatory activity whereas no significant activity could be detected at the low dose.

Discussion. The improvements and modifications made have eliminated the deficiencies of the original apparatus. Since it was observed that the aerosol mist concentrates at the periphery of the dome, the cage was designed to keep the animals as far as possible from the center. This modification increased the efficiency of the apparatus as shown by the reduced concentration of endotoxin (3 mg/ml instead of 12 mg/ml) required to produce maximum inflammatory response. The confinement of a small group of animals to each of the 4 sections of the cage undoubtedly contributed to this effect.

The study of the effects of various concentrations of endotoxin enabled us to demonstrate that within a certain range a direct relationship exists between concentration of endotoxin and the resulting increase in weight of the lung and degree of histopathological changes.

Attempts to prevent and/or reverse various types of experimentally induced inflammations with non-steroid substances at doses comparable (on a kilogram basis) to those used clinically have met with a certain degree of failure. From the literature, it appears that the method described by Wilhelmi

* Homogenous suspensions were obtained by means of a Tenbroeck grinder.

(2) and extensively used and standardized by Winder *et al*(3,4) is the only one sensitive enough to detect the anti-inflammatory activity of non-steroidal agents at doses comparable to the clinical ones. In brief, this method takes advantage of the ability of non-steroid agents, such as aspirin and phenylbutazone, to delay the development of the

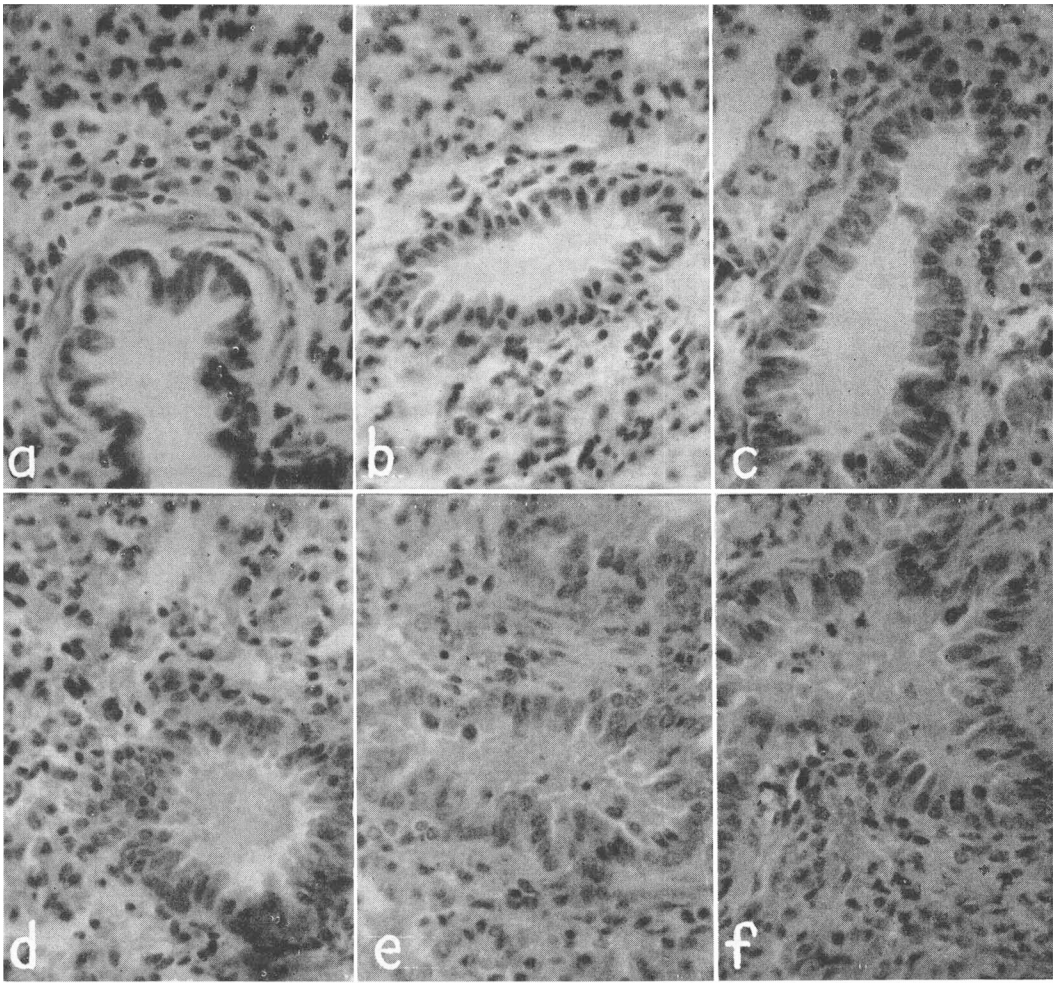

FIG. 4. Histological appearance of lungs of animals exposed to various concentrations of endotoxin—a) control; b) 0.11 mg/ml; c) 33 mg/ml; d) 1 mg/ml; e) 3 mg/ml; f) 12 mg/ml.

TABLE I. Anti-inflammatory Activity of Cortisone Acetate, Aspirin and Phenylbutazone as Determined by Reversal of the Increase in Lung Weight of Mice Exposed to 3 mg/ml Concentration of Aerosolized Bacterial Endotoxin.

Treatment	No. of animals*	Total dose (mg/kg)	Route of administration	% reversal	P
Cortisone acetate	30	20	s.c.	30.0	<.025
" "	30	40	"	47.9	<.001
Aspirin	35	20	p.o.	7.8	N.S.
"	35	60	"	40.0	<.025
Phenylbutazone	35	20	"	28.5	<.050
"	35	60	"	51.0	<.005

* No. of animals represents sum of 2 separate experiments.

ultraviolet-induced erythema in albino guinea pigs. However, even in this type of assay, treatment with the anti-inflammatory agent must precede the application of the ultraviolet light and furthermore, well-known anti-inflammatory steroids such as cortisone and hydrocortisone were ineffective(3).

Our method, based on reversal of endotoxin-induced lung inflammation in mice, has proven to be a reliable and sensitive assay for testing the anti-inflammatory properties of non-steroid as well as steroid agents. Some of the characteristics which make this anti-inflammatory assay more advantageous than other methods described in the literature have been mentioned previously(1). The method has been standardized and the minimum effective doses for cortisone acetate, aspirin, and phenylbutazone have been determined.

The minimum effective dose of cortisone acetate is comparable to that required to inhibit significantly the formation of the cotton pellet-induced granuloma. The minimum effective dose of phenylbutazone is comparable to that required to produce a significant delay in the development of ultraviolet erythema, whereas that of aspirin is considerably lower. It is noteworthy to mention that by using methods other than the ultraviolet erythema or that described in this paper oral doses up to 40 times as much of aspirin and phenylbutazone are required to produce a significant anti-inflammatory effect.

Summary. An improved anti-inflammatory assay based on the reversal of endotoxin-induced lung inflammation in mice is described. Changes in design of the original apparatus have: a) increased the number of animals which can be exposed to endotoxin; b) decreased the amount of endotoxin needed for maximum inflammatory response. A direct correlation exists between concentration of endotoxin and the resulting increase in weight of the lung and degree of histopathological changes. The advantages of this method for testing anti-inflammatory activity of steroid as well as non-steroid compounds are discussed in comparison with other methods described in the literature.

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Use of Primary Human Cell Cultures in Titering Certain Human Adenoviruses for Infectivity.* (29491)

JAMES D. CONNOR AND ALFREDO MARTI (Introduced by M. Michael Sigel)
Variety Children's Research Foundation, Miami, Fla.

There is a need for further development of sensitive methods for measuring the infectivity of the human adenoviruses, particularly for assays which may be used for all types. There have been frequent reports of titrations employing relatively short incubation times, although it is generally known that long periods of incubation may be required for demonstration of minute amounts of adenovirus either present in a "masked" form in the explanted tissues of the natural host or in tissue cultures as a result of inoculation.

During studies of mixed infections in tis-

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