Analysis of Dextran- and Methylated Albumin-Induced Hypersensitivity by Mouse Paw Swelling (34383)

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(Introduced by J. Doull)

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Numerous studies have been conducted on immediate and delayed hypersensitivity in mice. These studies have included skin tests, dye extravasation (1–5) and caliper measurements of paw swelling (6). Studies of hypersensitivity reactions in mice are particularly suitable because of the large number of inbred strains available and the economy of maintenance. Van Arman *et al.* (7) and Winter *et al.* (8) have utilized a plethysmographic measurement for the study of irritative agents on swelling of foot paws of rats. This procedure also provides a novel, quantitative, and delicate method for studying dermal allergic reactions in mice, as was shown by the present studies which were designed to answer two questions: Can plethysmographic measurements be performed with precision and reliability on volumes as small as mouse paws; will immunological swelling of mouse paws be of sufficient magnitude to enable one to quantitatively assess the degree of reactivity? In large measure these questions have been answered in our present study.

The present communication deals with (i) the application of the plethysmographic method to the study of small volumes, (ii) experiments dealing with the precision and reliability of the method, and (iii) the application of the method to the study of mouse paw swelling induced by dextran and methylated human serum albumin (MHSA), agents which produce edema as a result of previous immunization. This technique permits one to distinguish between swelling induced by a toxic or irritative component and swelling immunologically induced by an agent. Recently, Axelrad (9) described an assay for delayed hypersensitivity by immersing rat paw in a mercury-filled beaker placed on a top-loading balance.

Materials and Methods. Male LAF_1 (C57L $\times$ A/He) mice purchased from Jackson Memorial Laboratories, Bar Harbor, Maine, 6–16 weeks of age, were used for these experiments. Native dextran, 5–40 million in molecular weight, and crystalline human serum albumin were purchased from Nutritional Biochemicals, Cleveland, Ohio. Methylated human serum albumin (MHSA) was prepared according to the procedure of Crowle and Hu (5). Microliter syringes for subplantar injections were purchased from Hamilton Co., Whittier, California.

Plethysmograph measurement of small volumes. The plethysmographic instrumentation was a modification of the method of Van Arman *et al.* (7). The apparatus consists of a plastic cup filled with mercury in which mouse paws are immersed; small pressure changes, caused by displacement of the mercury level by the immersed object, are proportionally converted by a low-pressure transducer to electrical signals which are recorded on a chart recorder. The instrument consisted of the following: (i) Gilson model M5 polygraph, Gilson Medical Electronics, Middleton, Wisconsin. (ii) Statham model P23BB transducer, 0.5 cm Hg, Statham Laboratories, Inc., Hato Rey, Puerto Rico. (iii) Plastic cup of 12 mm i.d. The size of the cup was of a convenient diameter to allow for paw immersion without touching the sides of the cup.

In order to assess the reliability of the method for small volumes, stainless steel cylinders of various diameters were employed and were marked at 2.5- and 5.0-mm intervals. Six replicate readings were made for each volume increment of the standards.

Mouse paw measurements. To measure edema, 20 μl of the agent in saline were

injected into the subplantar region of the left hind paw. Twenty μl of saline injected into the right hind paw served as a control. The solutions were injected using 27-gauge needles and Hamilton microliter syringes. In early expermients animal movement was prevented by pentobarbital anesthesia when their paws were being immersed in the mercury bath; but later, mice were hand-held without anesthesia. Each hind paw was marked with ink at the femoral tibial joint so that readings could be made by immersing a paw into the mercury reservoir to the same depth. Each paw was measured six times in 20–30 seconds. Every value in Figs. 2–5 is the average of left minus right paw recordings. Pen deflections on Gilson polygraph charts were read to the nearest 0.1 mm.

Immunization of mice with dextran and methylated human serum albumin (MHSA). On the basis of some preliminary studies dealing with dosage and time intervals of immunization, the following schedules were adopted: (i) For dextran immunization, each mouse was given a single subscapular injection of dextran (1.0 mg in 0.1 ml of saline). On the day of paw assay, *i.e.*, 1, 2, 3, and 4 weeks after immunization a soluble challenge antigen (200 μg of dextran in 20 μl of saline) was injected in the subplantar region of the hind paw. Twenty μl of saline in the other paw served as control. (ii) When employed, incomplete Freund adjuvant (I.F.A.) was mixed with an equal volume of dextran and 0.2 ml of the mixture was injected into each mouse. On the day of paw assay, a soluble challenge antigen was injected in the hind paw as in (i) above. (iii) For methylated human serum albumin (MHSA) immunization, each mouse was injected twice (1 week apart) with dextran (1.0 mg of MHSA in 0.1 ml of water). On the day of paw assay, *i.e.*, at 2, 3, and 4 weeks after immunization, a soluble challenge antigen (200 μg of MHSA in 20 μl of water) was injected in the subplantar region of the hind paw. Twenty μl of water injected in the other paw served as control. (iv) When employed, IFA was mixed with an equal volume of MHSA and 0.2 ml of the mixture was injected into each mouse. On the day of paw assay, a soluble challenge antigen was injected in the hind paw as in (iii) above.

Results. Measurement of small volumes. In order to assess the sensitivity of the plethysmographic technique for measurement of small volumes, various sized stainless steel cylinders were employed as standards. The results of these studies are shown in Fig. 1. The diameters of the standards used were (mm): 3.17 (Fig. 1a), 3.95 (Fig. 1b), and 4.76 (Fig. 1c). Volumes expressed as μl were calculated on the basis of $\pi r^2 h$ for each increment of the standards immersed. Each standard was marked with a felt pen and hand-held for immersion to approximate conditions which would prevail when mouse paws would be immersed in mercury reservoirs. Each point in Fig. 1a and b represents pen deflections on a Gilson MP5 polygraph resulting from immersion of 5-mm increments of standards into the mercury bath. Each point in Fig. 1c represents pen deflections resulting from immersion of 2.5-mm incre-

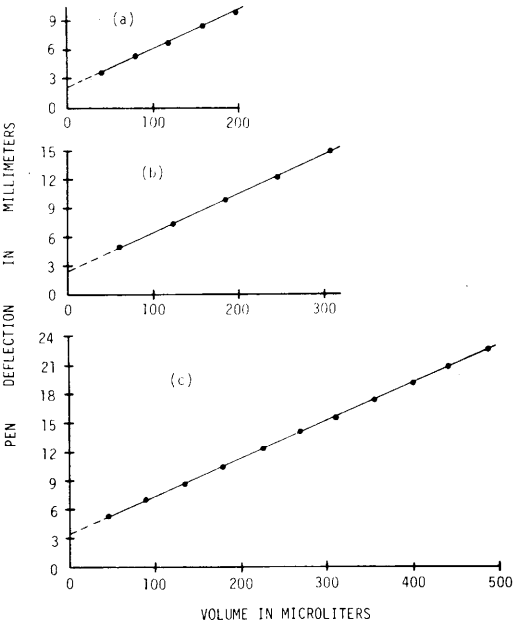

FIG. 1. Comparison of pen deflection readings with volumes of stainless steel cylinders immersed in mercury reservoirs. Diameters of cylinders were (mm): (a) 3.17; (b) 3.95; and (c) 4.76. Each point in (a) and (b) represents 5.0-mm increment of cylinders immersed; each point in (c) represents 2.5-mm increment of the cylinder immersed.

TABLE I. Calculation of Volumes from Immersion of 20-mm Lengths of Standards.[a]

(Fig. 1)	(a) Diameter of standards (mm)	(b) Pen deflection/ 20-mm length of standard (mm)	(c) Calc vol of 20-mm standard[a] (μl)	(d) Calc vol/mm of pen deflection (μl)
a	3.17	6.2	158	25.48
b	3.95	9.6	245	25.52
c	4.76	13.9	355	25.54
Av				25.51

[a] Calculated on the basis of $\pi r^2 h$ or $\pi r^2 \cdot 20$.

ments of the standard. Also, each point in the 3 graphs represents the mean of 6 replicate readings; standard errors of replicate readings for each point were negligible and could not be drawn in the graphs.

As shown in Fig. 1a, b, and c, regardless of the range of volumes, there was linearity throughout the entire range of volumes measured, although the lines did not go through the point of origin. As larger standards were used, the applied force necessary to "wet" the mercury was larger; this is shown by correspondingly larger pen deflections (compare dotted lines intersecting ordinates of Fig. 1a, b, and c).

In Table I are shown calculated volumes from immersion of 20-mm lengths of the 3 standards. Volumes of 158, 245, and 355 μl were calculated from readings between 5 to 25 mm of standards immersed (Col. c). Volumes of this length were used to obviate the necessity of considering the applied force necessary to "wet" mercury; also this length approximated the immersed length of mouse paws. In Column b are recorded pen deflections resulting from immersion of 20 mm lengths of the standards. Column d indicated that close correspondence existed between the 3 standards; the mean of the 3 standards indicated that 1 mm of pen deflection corresponds to 25.5 μl of volume. This indicated that regardless of the diameter of the immersed object, the volume represented by each mm of pen deflection was the same.

It was important to assess the minimum volume which can be detected by the plethysmographic technique (Table II). A stainless steel cylinder, 2.38 mm in diameter, was used for this determination. Six replicate readings were made of the standard immersed at 10-, 12-, and 13-mm mark in the mercury bath. The volumes immersed were converted from millimeters of pen deflection to microliters of volume on the basis of 25.5 μl/mm of pen deflection. Thus, lengths of 10, 12, and 13 mm of the standard represented 44.5, 53.4, and 57.8 μl immersed in the mercury bath. Two-mm lengths of the standard represented 8.9 μl of volume (53.4–44.5) whereas a 1-mm length represented a volume of 4.4 μl (57.8–53.4). On the basis of 6 replicate readings for each volume measured, the diff-

TABLE II. Statistical Significance between Readings at 10 and 12 mm Immersion and 12 and 13 mm Immersion of a Stainless Steel Cylinder of 2.38 mm in Diameter.

(a) Length of standard immersed (mm)	(b) Significance	(c) Vol of standard immersed (μl)	(d) Vol change (μl)
10	$p < 0.001$	44.5	8.9
12		53.4	
12	$p < 0.01$	53.4	4.4
13		57.8	

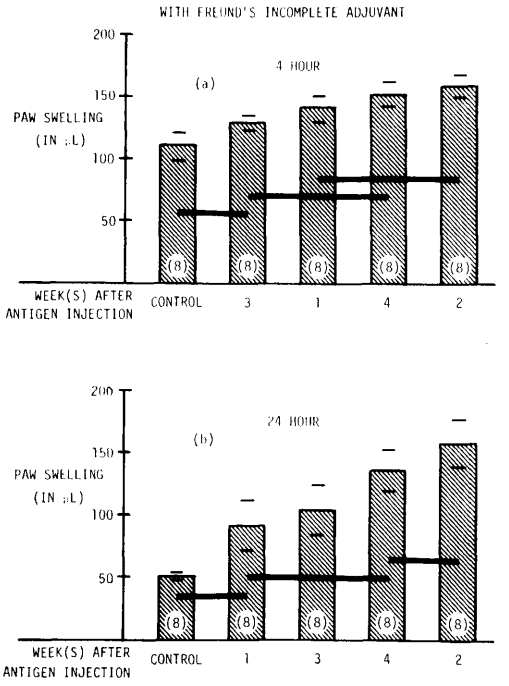

FIG. 2. Paw swelling resulting from a subplantar injection of 200 μg of dextran in LAF_1 mice previously immunized with dextran–incomplete Freund's adjuvant mixture. Distance between lines of each bar graph represent two standard errors of the mean. Bar graphs connected by a horizontal line are not statistcally different from each other but are different from all other values not connected by the horizontal line. Number of animals in each experiment is in parentheses. (a) represents values obtained at 4 hr and (b) represents values obtained at 24 hr after subplantar injections.

erences between the readings were significantly different according to Student's t test distribution. Readings at 12 and 13 mm of length immersed, representing a volume difference of 4.4 μl, were significantly different at the 0.01 level of significance (Column b). Hence, present data indicate that lengths of 12 and 13 mm, representing volume differences of 4.4 μl, can be distinguished by this technique.

Assessment of immunologically enhanced swelling of mouse foot paws. The preceding studies established a firm basis for a study of agents which promote volume changes in appendages as small as mouse paws. The aim of our present studies was to quantitatively assess the degree of paw swelling resulting from prior injections of antigen which can bring about swelling in mouse paws given subplantar injections of antigen. That this can be accomplished is shown by the results in Figs. 2 and 3. Previously, Crowle and Hu (5) demonstrated hypersensitivity to dextran by skin tests in mice.

In our experiments, animals were given a single subscapular injection of dextran with or without incomplete Freund adjuvant (IFA). Different groups of 4 animals were assessed for paw swelling at various weeks after antigen injection (see *"Material and Methods"* for antigen injection schedule). The experiment was run twice and the combined data from two experiments were analyzed statistically. Therefore, each bar graph in Figs. 2 and 3 represents mean $\pm$ standard error of 8 animals. Controls represent animals which were not injected with antigen. The data were analyzed by Duncan's new multiple range test preceded by an analysis of variance (10). Interactions between replicate readings for each determi-

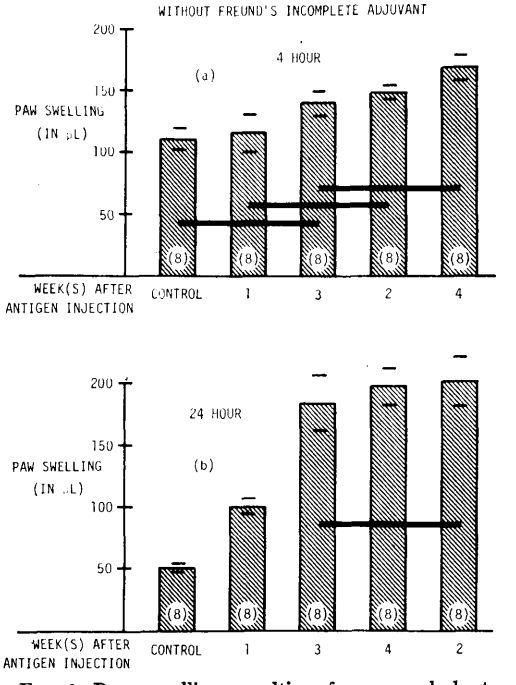

FIG. 3. Paw swelling resulting from a subplantar injection of 200 μg of dextran in LAF_1 mice previously immunized with dextran alone; see legend in Fig. 2 for other details.

nation were not significantly different from each other; nor were values for animals within each group significantly different ($p>0.05$). In Figs. 2 and 3, bar graphs which are connected by a horizontal line are not significantly different from each other but are significantly different from all other values not connected by the horizontal line (employing Duncan's new multiple range test at the 0.05 level of significance). Figure 2 represents experimental studies in which LAF_1 mice were given subscapular injections of antigen in a 1:1 mixture with IFA and groups of animals were analyzed for paw swelling at 1, 2, 3, and 4 weeks after antigen injections. Preliminary experiments indicated that the toxic or irritative component is maximal at 4 hr following subplantar injections and delayed swelling occurs around 24 hr after subplantar injections in a primed animal. We have tentatively assigned these two time intervals as representing early and late responses of the animals to the antigen. Comparing control values of 4- and 24-hr readings in Fig. 3, the bar graphs indicate that dextran, by itself, has considerable edema-producing properties (100 μl). In 24 hr, the amount of paw swelling, however, is reduced to 50 μl.

The results from Fig. 2a indicated that although paw swelling at 3 weeks was not significantly different from control values, groups which were analyzed at 1, 4, and 2 weeks following antigen injection were significantly different from controls. Thus, swelling is evident in the early response and is dependent on prior immunization with antigen. A clearer distinction between control and experimental groups is evident at 24 hr. Data, shown in Fig. 2b, indicated that, with the exception of the 1-week group, all other groups were significantly different from the control group. A threefold increase in paw swelling was demonstrated at 2 weeks following antigen injection, whereas groups analyzed at 3 and 4 weeks following antigen showed greater than twofold increase in swelling.

A similar dextran injection scheme in LAF_1, but without IFA, was carried out. These studies, shown in Fig. 3, indicated a similar pattern of response although the magnitude of differences in paw swelling at 24 hr after subplantar injections was greater than for those injected with IFA (Compare Fig. 2b and 3b).

Hypersensitivity induced with methylated human serum albumin. Since a large degree of paw edema associated with the irritative component of dextran tended to obscure swelling associated with the immune component, substances lacking the irritative component were sought. Methylated albumin used by Crowle and Hu (5) for skin sensitization assays, was found to be a suitable agent. The results of these studies are shown in Fig. 4 (with IFA) and 5 (without IFA). In Figs. 4 and 5, bar graphs which are connected by a horizontal line are not significantly different from each other but are significantly different

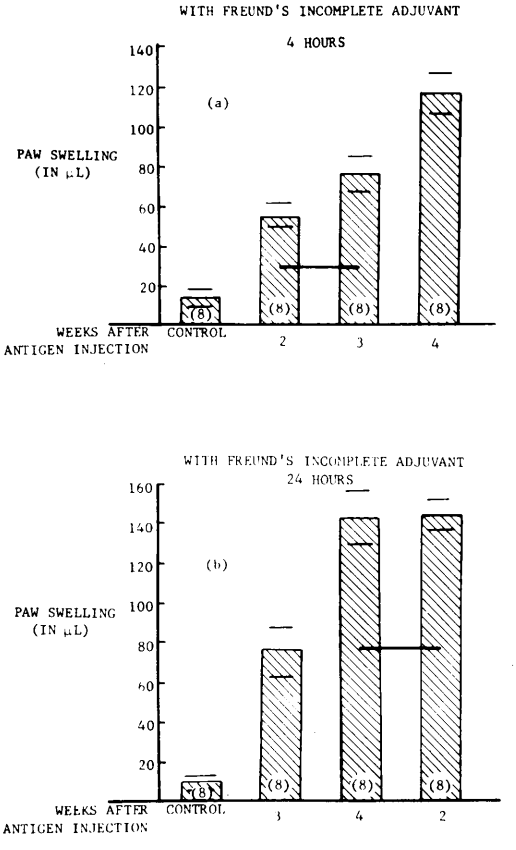

FIG. 4. Paw swelling resulting from a subplantar injection of 200 μg of MHSA in LAF_1 mice previously immunized with MHSA–incomplete Freund's adjuvant mixture; see legend in Fig. 2 for other details.

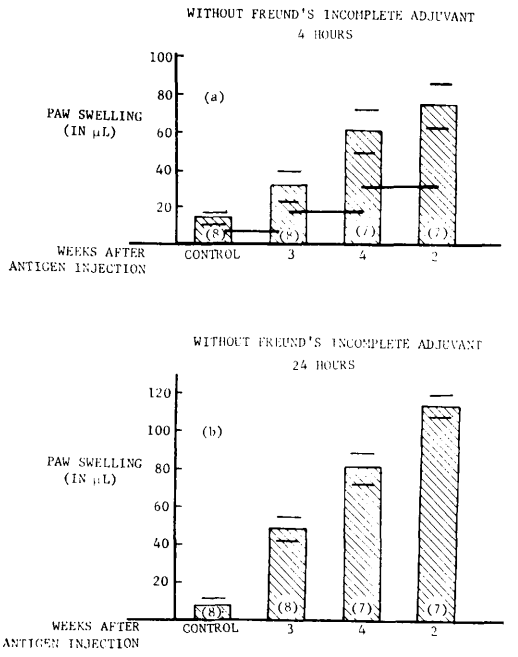

FIG. 5. Paw swelling resulting from a subplantar injection of 200 μg of MHSA in LAF_1 mice previously immunized with MHSA alone; see legend in Fig. 2 for other details.

from all other values not connected by the horizontal line (10). The results indicated that control animals showed little of the irritative component, showing less than 20 μl at 4 hr and even less at 24 hr.

In animals receiving MHSA with IFA, observed swelling at 4 hr increased weekly with maximal values attained at 4 weeks. The pattern of response noted at 24 hr following subplantar injections indicated that maximal swelling, observed at 2 and 4 weeks after the last priming injection, was significantly higher than that observed at 3 weeks. In mice immunized with MHSA without IFA (Fig. 5), a pattern of response observed at 24 hr after subplantar injections was similar although the magnitude of paw swelling was lower than that observed with IFA. In addition, swelling observed at 3 weeks was lower than 2- and 4-week values (Fig. 5b).

Discussion. Our studies have demonstrated several advantages of the plethysmographic technique over other techniques in assessing hypersensitivity in mice. Use of different sized stainless steel cylinders as standards has demonstrated the validity of the plethysmographic technique for the study of small volume changes as occur in mouse paws. Replicate readings can be made quickly and can be reproduced with minimum practice. Comparisons of antigenic potency, at various time intervals after immunization, can be analyzed statistically. Measurements of reactivity can be made over protracted periods of time without killing the animals to assess the degree of reactivity.

Since dextran possessed a toxic or irritative component which produced approximately 100 μl of edema 4 hr following subplantar injections in nonimmunized control animals, immunologically- enhanced paw swelling was minimized by the large irritative component; it was more suitable in studies of the 24-hr response since the irritative swelling in nonimmunized controls had decreased to values of 50 μl. Methylated human serum albumin, because of its low degree of irritative swelling, viz., less than 20 μl, is preferred over dextran in the present studies. An unexpected finding was that in all injection schemes but one, for dextran and MHSA, the swelling response was lower at 3 weeks than at 2 and 4 weeks after the last subscapular injection. The reasons for the temporally-related biphasic response to the challenge antigen are not presently known.

The injection schemes adopted for the present studies were simple and were used to demonstrate maximal responses of hypersensitivity to the two antigens. Other schemes which may show a clearer distinction between the 4- and 24-hr response are currently under investigation. In their skin sensitization assays Crowle and Hu (11) have assigned 3- and 24-hr intervals as representing Arthus and delayed hypersensitivity reactions. Whether paw induration, observed at 4 and 24 hr after subplantar injections of antigen in previously immunized animals, is due to immediate and delayed hypersensitivity remains to be determined.

Summary. Dermal allergic reactions in LAF_1 mice arising from prior immunizations with dextran or methylated albumin were studied. A plethysmographic technique for

measuring mouse paw swelling was developed. At weekly intervals after immunization, mouse paw swelling was assessed to determine the degree of allergic swelling. Because of the large irritative swelling component of dextran itself, methylated albumin was more suitable for studying early and late dermal allergic responses. In all injection schemes but one, for dextran and methylated albumin, mouse paw swelling was lower at 3 weeks than at 2 and 4 weeks after the last immunization.

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