

Rates of Release of Subcutaneously Injected Antigens in the Rat

Comparison of an Aqueous Preparation with Two Alum-Precipitated Preparations

JERRY L. LEELING, INDU A. MUNI*, RICHARD J. HELMS AND NED JOHNSON, JR.

Toxicology Department, Corporate Research Miles Laboratories, Inc., Elkhart, Indiana, U.S.A.

Release of antigen E-¹²⁵I from the site of subcutaneous injection in male rats was delayed significantly when either of two alum-precipitated preparations containing 1.0-1.2 mg of Al/ml was administered rather than an aqueous preparation. The rates of ¹²⁵I excretion were similarly influenced being statistically slower through the first week after single doses of the alum-precipitated preparations. The results of these studies strongly support the view that alum-precipitated vaccines offer more protection from system reactions than aqueous vaccines.

Key words: allergens; alum-precipitated; aqueous; rat; release rates.

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For several years alum-precipitated pyridine extracts have been used in treatment of various allergic conditions (1, 2, 5, 8, 10, 11, 12). While it is generally recognized that use of alum-precipitated extracts results in delayed absorption of antigen from the site of injection, little is really known concerning the rate of antigen release. Holtzman & Norman (4) in a preliminary experiment demonstrated that the rate of release of ¹³¹I-labeled human serum albumin from a subcutaneous site in the rabbit hind foot was an inverse function of the alum concentration. However, the alum concentrations used in their study were relatively high. In the study reported below the rates of absorption of ¹²⁵I-labeled antigen E after subcutaneous administration of two Fraction A alum-precipitated extracts** were compared

to that of an aqueous Fraction A extract** in the rat.

MATERIAL AND METHODS

Labeled antigens

Purified antigen E (Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.) was iodinated by the chloramine-T method (3) using Na¹²⁵I (New England Nuclear, Boston, Massachusetts, U.S.A.).

A 10% low ragweed extract was prepared by ultrafiltration and the retentate was lyophilized. A portion of this aqueous ragweed extract was dissolved in isotonic saline and antigen E-¹²⁵I was added resulting in a preparation with an ¹²⁵I specific activity of 0.98 μ Ci/1000 P.N.U.

A 100-mg aliquot of lyophilized aqueous preparation was dissolved in 20 ml of saline. Fifteen ml of this preparation was incubated for 2 h at room temperature with 15 ml of autoclaved preformed alum (saline-washed

* Present address: Industrial Bio-Test, Northbrook, Illinois, U.S.A.

** Alpare (alum preparation I), Allpyral (alum preparation II), and Aquare (aqueous preparation) are registered trademarks of Dome Laboratories Division, Miles Laboratories, Inc., West Haven, Connecticut (U.S.A.).

precipitate obtained by adjusting a 2% aluminum potassium sulfate solution to pH 6.5 with NaOH) in saline suspension. The alum precipitate was washed 3 times with an isotonic saline solution containing 0.4% phenol, then resuspended to 15 ml in phenol-saline. A 1.75-ml aliquot of antigen E-¹²⁵I solution was also incubated with autoclaved preformed alum, then washed and resuspended in phenol-saline (alum preparation I). Ten-ml aliquots of this suspension and alum-adsorbed antigen E-¹²⁵I were mixed. The resulting suspension contained 1.0 mg of alum/ml and had an ¹²⁵I specific activity of 1.4 μ Ci/1000 P.N.U.

A 5-ml aliquot of antigen E-¹²⁵I was incubated overnight in equal parts of 0.1 N NaOH-50% pyridine. The precipitate which formed after the addition of 10 ml water and 11 ml of 2% aluminum potassium sulfate in 0.25 N sulfuric acid was washed 4 times with 30 ml of phenol-saline. This alum-adsorbed antigen E-¹²⁵I was finally suspended in 15 ml of phenol-saline and mixed with 7.5 ml of commercial alum preparation II (Dome Laboratories Lot No. 601044 CY; 20,000 P.N.U./ml). The resulting suspension of alum preparation II contained 1.2 mg of alum/ml and had an ¹²⁵I specific activity of 1.17 μ Ci/1000 P.N.U.

Animals

A total of 102 (including three control animals) male Charles River COBS-CD rats, weighing 150–195 g initially, were used in these experiments. All rats were housed in individual stainless steel metabolism cages and received food and water *ad libitum* throughout the experimental period.

Experimental design

In each of three separate experiments 33 rats were assigned at random (6) to nine groups of three animals each and one group of six. In the first experiment all rats received by single subcutaneous (interscapular) injection 0.2 ml containing 1000 P.N.U. of ¹²⁵I-labeled alum

preparation I. In the second and third experiments 1000 P.N.U. doses of ¹²⁵I-labeled alum preparation II and aqueous extract, respectively, were administered in identical fashion. Three rats from each experimental group were sacrificed by ether inhalation at intervals of 10 min, 1, 4, 8, 24, and 48 h, 1, 4, and 6 weeks after injection. Tissues removed from each of these rats at death were: the injection site (interscapular skin and all underlying non-osseous tissue, about one sq. in. of each), brain, kidneys, liver, lungs, mesenteric lymph nodes, plasma, spleen, and thyroid. Urine and feces were collected daily through 42 days after dosing from the remaining six animals in each experimental group. Three additional rats were used to provide control samples of tissues and excreta for all three experiments.

Tissue and urine samples were stored frozen for subsequent analysis. Feces were dried, powdered, and weighed. In order to determine the amount of radioactivity remaining in rats after 6 weeks, two rats from each excretion study were killed, pulverized in a meat grinder, and desiccated. Radioactivity in tissue, urine, feces, or aliquoted whole animal was determined by gamma counting using a Packard Tri-Carb Model 5023-Model 3380 tandem gamma spectrometer (Downers Grove, Illinois, U.S.A.). All samples were counted for 10 min or 10,000 counts. Counting efficiency (75–83%) was determined daily by counting an ¹²⁵I standard and all c.p.m. were converted to d.p.m.

The statistical significance of differences in amounts of ¹²⁵I remaining at subcutaneous injection sites, or found in excreta of rats after treatment with the three antigen E-¹²⁵I-containing allergenic products, was determined by application of Scheffe's multiple *t* test (7). Areas under tissue ¹²⁵I concentration (d.p.m./g)-time curves were determined by the trapezoidal method of integration.

RESULTS

The rates of ¹²⁵I release from the site of subcutaneous injection of a single 1000 P.N.U.

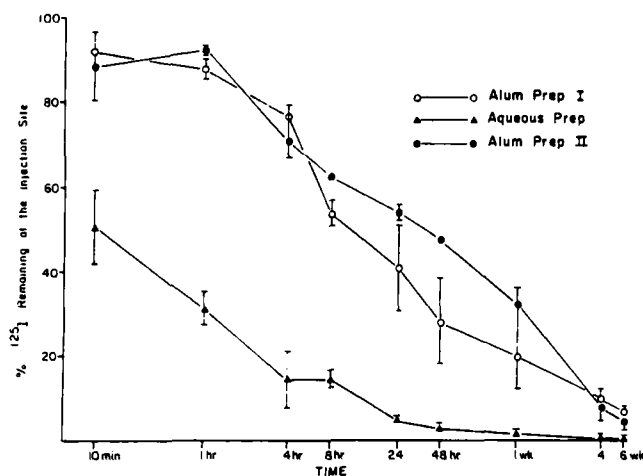


Fig. 1. The release rate of ^{125}I -labeled ragweed antigen E in aqueous solution or adsorbed to aluminum hydroxide. Vertical bars show standard error of the mean.

dose of ragweed extract preparation containing antigen E- ^{125}I to rats are illustrated in Fig. 1. These results show clearly that both alum-precipitated preparations effectively delayed absorption of ^{125}I from the injection site, while 50% of the ^{125}I in the aqueous preparation had already been absorbed 10 min after injection. Statistically significant differences between ^{125}I remaining at the sites of injection of the aqueous and the two alum-precipitated ragweed extracts were observed through the first week.

As indicated in Table 1, the distribution of

^{125}I in tissues was qualitatively not much different from preparation to preparation. These data are not presented quantitatively because, although tissue curve areas were generally higher in the first 24 h after administration of the aqueous preparation, the areas estimated were skewed heavily in favor of the alum-precipitated preparations. The above occurred because 50% of the ^{125}I in the dose of aqueous preparation escaped detection in the first 10 min, presumably passing into the urinary bladder. Plasma levels of ^{125}I following a single dose of three preparations are illustrated in Fig. 2. Plasma levels, while not necessarily representative of levels in the other tissues studied, reflect the speed of release of ^{125}I from the site of injection of the aqueous ragweed extract.

The excretion of ^{125}I by rats in the 6-week period after a single subcutaneous dose of ^{125}I -containing allergens is illustrated in Fig. 3. The distribution of ^{125}I between urine and feces (expressed as % of dose) was 81.88 urinary/10.62 fecal, 89.30/9.29, and 80.78/8.32 on the 42nd day after administration of the aqueous preparation, alum preparation I, and alum preparation II, respectively.

Since the percentages of total ^{125}I excreted varied slightly from preparation to preparation, all individual data in each group were

Table 1

Rat tissues ranked according to relative 24-h concentration of ^{125}I

Order of decreasing area ^a under tissue ^{125}I level-time curves		
Aqueous prep.	Alum prep. I	Alum prep. II
Thyroid	thyroid	thyroid
Kidneys	kidneys	kidneys
Plasma	plasma	plasma
Spleen	lungs	spleen
Liver	spleen	lungs
Lungs	liver	liver
Lymph nodes	lymph nodes	lymph nodes
Brain	brain	brain

^a Areas expressed as d.p.m. of ^{125}I /g tissue $\times$ time in hours (0–24 h data used).

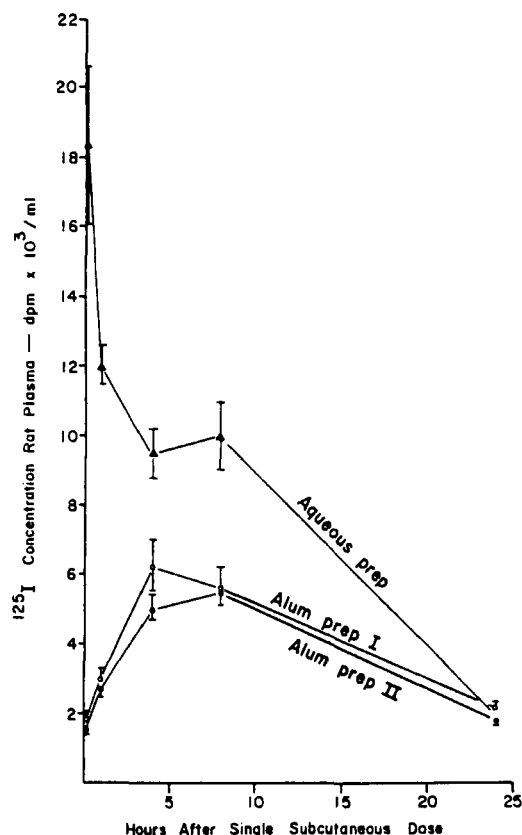


Fig. 2. Plasma levels of ^{125}I in the rat during first 24 h after a single subcutaneous dose of ^{125}I -labeled hyposensitizing vaccines. Vertical bars show standard error of the mean.

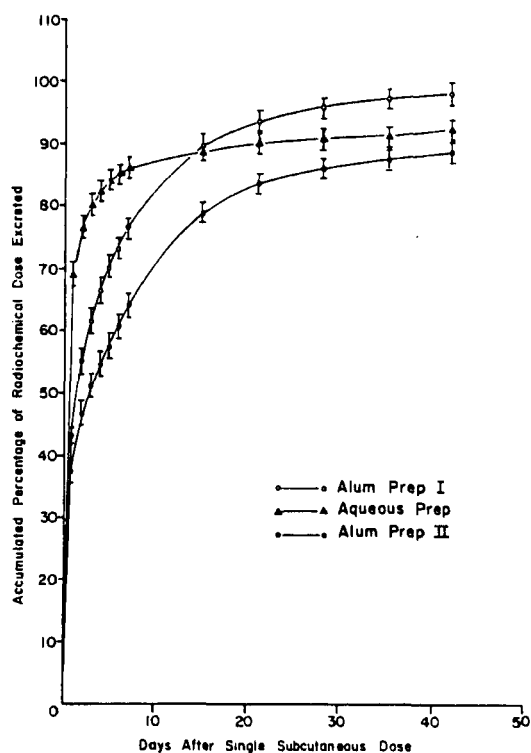


Fig. 3. Excretion of ^{125}I in 6-week period after a single subcutaneous dose of ^{125}I -labeled hyposensitizing vaccines. Vertical bars show standard error of the mean.

Table 2

Percentage of total ^{125}I excreted by rats at various intervals after a single subcutaneous dose of aqueous or alum-precipitated preparations each containing antigen E- ^{125}I

Day	Percentage of 42-day total excreted on indicated day ^a		
	Aqueous Prep.	Alum Prep. I	Alum Prep. II
1	74.58 $\pm$ 2.05	43.72 $\pm$ 1.19	41.99 $\pm$ 1.94
4	88.95 $\pm$ 1.68	67.47 $\pm$ 2.05	61.54 $\pm$ 2.23
7	92.69 $\pm$ 1.61	77.63 $\pm$ 1.80	72.07 $\pm$ 2.09
14	95.78 $\pm$ 1.69	91.09 $\pm$ 1.84	88.73 $\pm$ 1.81
21	97.16 $\pm$ 1.69	95.10 $\pm$ 1.85	93.95 $\pm$ 1.84
28	97.98 $\pm$ 1.71	97.42 $\pm$ 1.78	96.83 $\pm$ 1.91
35	98.58 $\pm$ 1.76	98.95 $\pm$ 1.73	98.52 $\pm$ 1.96
42	100.00 $\pm$ 1.78	100.00 $\pm$ 1.78	100.00 $\pm$ 2.03

^a Mean $\pm$ s.e.m. ($n=6$). Means *not* statistically different are italicized.

Table 3

Recovery of ^{125}I from rats following a single subcutaneous dose of aqueous- or alum-precipitated preparations each containing antigen E- ^{125}I

Treatment	Recovered in excreta	Remaining in carcass	Total recovered
Aqueous prep.	94.04 $\pm$ 5.77	2.35 $\pm$ 0.39	96.39 $\pm$ 6.16
Alum prep. I	97.12 $\pm$ 5.02	1.79 $\pm$ 0.57	98.91 $\pm$ 5.54
Alum prep. II	89.35 $\pm$ 5.88	3.58 $\pm$ 0.98	92.93 $\pm$ 6.86

* Mean $\pm$ s.e.m. ($n=2$).

reexpressed as percentages of the group mean 42-day total percentage. These data (Table 2) were subjected to statistical evaluation which revealed that accumulated ^{125}I excretion after administration of aqueous extract was statistically greater than after either alum-precipitated preparation the first week. Thereafter, however, there were no statistical differences between the preparations.

The recovery of ^{125}I from two rats in each treatment group is summarized in Table 3. Average total recovery ranged from about 93% of the dose in alum preparation II-treated rats to about 99% in rats treated with the aqueous preparation. Differences between dose groups were not statistically significant.

DISCUSSION

The results of this study in rats are not consistent with earlier observations in rabbits (4,9). Holtzman & Norman (4) were able to demonstrate delayed release of allergen only at alum concentrations much higher than those used in the present study. Talmadge & Dixon (9) using alum concentrations similar to the present study (i.e., about 1 mg/ml) observed release rates only slightly slower than that of the aqueous ragweed extract.

The direct evidence for delayed release of ^{125}I from the site of injection of alum-precipitated extracts was corroborated by the rates of excretion of ^{125}I after those preparations in relation to that observed after the

aqueous preparation. In addition, plasma levels of ^{125}I after administration of the aqueous extract resembled those which would be expected if an intravenous injection had been given. Plasma levels of ^{125}I which were observed after administration of the alum-precipitated preparations were descriptive of slowly absorbed material. The high thyroid and kidney levels of ^{125}I probably reflected the roles of those tissues in the sequestration and excretion (about 90% of the ^{125}I excreted was passed in the urine) of ^{125}I , respectively. The recovery of ^{125}I from the excreta of rats was essentially complete 42 days after each of the preparations. However, a small quantity of ^{125}I was found in the body at that time. The location of this material was not determined but it is doubtful that any intact antigen E- ^{125}I remained.

Although only antigen E was labeled in the present study and it is well known that ragweed extracts contain several other antigens, antigen E is of major importance and seems fairly representative of ragweed antigens in general. In view of the extreme differences between the aqueous and alum-precipitated preparations in release rate of antigen E- ^{125}I from the injection site, particularly in the critical first hour after injection, it seems safe to conclude that the results of these studies strongly support the view that alum-precipitated ragweed pollen vaccines offer considerable advantage over aqueous hypo-sensitizing preparations in terms of freedom from system reactions.

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REFERENCES

1. Ahlstedt, S. & Eriksson, N. E.: Immunotherapy in atopic allergy-antibody titres and avidities during hypo-sensitization with birch and timothy allergens. *Int. Arch. Allergy Appl. Immunol.* **55**, 400-411, 1977.
2. Fuchs, A. M. & Strauss, M. B.: Clinical evaluation, preparations and standardization of suspension of a new water-insoluble whole ragweed pollen complex. *J. Allergy* **30**, 66-82, 1959.
3. Greenwood, F. C., Hunter, W. M. & Glover, J. S.: The preparation of ^{125}I -labelled human growth hormone of high specific radioactivity. *Biochem. J.* **89**, 114-123, 1963.
4. Holtzman, R. S. & Norman, P. S.: The effect of alum concentration on the respository and adjuvant properties of alum-precipitated antigens. *J. Allergy* **38**, 65-73, 1966.
5. Lichtenstein, L. M., Norman, P. S. & Winkenwerder, W. I.: Antibody response following immunotherapy in ragweed hay fever: Allpyral vs whole ragweed extract. *J. Allergy* **41**, 49-57, 1968.
6. Snedecor, G. W. & Cochran, W. G.: Statistical methods, 6th edn., p. 69, Iowa State University Press, Ames, Iowa, 1969.
7. Scheffe, H.: The analysis of variance, p. 73. John Wiley & Sons, New York, 1967.
8. Spiegelman, J., Friedman, H. & Tuft, L.: Immunologic responses of pollinosis patients treated with alum-precipitated ragweed extract. *Ann. Allergy* **25**, 262-274, 1967.
9. Talmage, D. W. & Dixon, F. J.: The influence of adjuvants on the elimination of soluble protein antigens and the associated antibody responses. *J. Infect. Dis.* **93**, 176-180, 1953.
10. Tuft, L. & Friedman, H.: Prolonged immunotherapy with alum-precipitated pyridine ragweed extracts - a clinical and immunological study. *Ann. Allergy* **39**, 426-430, 1977.
11. Tuft, L., Spiegelman, J. & Friedman, H.: Serologic responses to alum-precipitated pyridine extracted ragweed pollen in allergic patients. *Ann. Allergy* **26**, 552-560, 1968.
12. Tuft, L., Spiegelman, J., Stupniker, S., Brown, E., Torsney, P. J. & Gilday, F.: The use of alum-precipitated pyridine pollen extracts in the treatment of ragweed pollinosis. *Am. J. Med. Sci.* **250**, 668-674, 1965.

Address:

Jerry L. Leeling
 Toxicology Department
 Corporate Research
 Miles Laboratories, Inc.
 P.O. Box 40
 Elkhart, IA 46515
 U.S.A.