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QUANTITATIVE ANALYSIS OF ALLERGENS BY A PASSIVE TRANSFER METHOD AS DEMONSTRATED WITH FRACTIONS OF COTTONSEED ALLERGEN, CS-1A

QUANTITATIVE MEASUREMENT OF THE MIGRATION OF INTRACUTANEOUSLY INJECTED COTTONSEED ALLERGEN IN PASSIVE TRANSFER STUDIES

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QUANTITATIVE ANALYSIS OF ALLERGENS BY A PASSIVE TRANSFER METHOD AS DEMONSTRATED WITH FRACTIONS OF COTTONSEED ALLERGEN, CS-1A

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INTRODUCTION

THE isolation and properties of the principal allergen of cottonseed, CS-1A, have been described in a series of papers from this laboratory starting in 1939.[1] CS-1A was immunologically distinct from other allergens[2, 3] and antigens[4] in the cottonseed and was classified as a natural proteose.[4, 5] The protein nature of CS-1A was shown by chemical properties, isolations procedures, amino acid composition,[6] loss of activity on proteolysis,[7] and antigenicity.[8]

In a further study of CS-1A, dialysis and ion-exchange fractionation were used.[9] Eighteen subfractions were obtained from each of the dialysate (D series) and the endo (E series) fractions. Two fractions which represented maximum separation by these methods, (CS-13D)1A and (CS-13E)5F6, were completely free of each other as shown by an ion-exchange method of characterization and by a precipitin method.[9]

The purpose of this article is to evaluate the allergenic relationships of (CS-13D)1A, (CS-13E)5F6, and other important fractions from CS-1A and to describe a method for quantitatively comparing three properties of two allergenic fractions by the passive transfer technique. The properties are (1) the relative passive-transfer-inciting capacities, (2) the relative reagin-neutralizing capacities, and (3) cross-neutralization to determine whether or not the two fractions have the same specificity.

Walzer and Kramer,[10] in 1925, first adapted the passive transfer reaction of Prausnitz and Küstner[11] to diagnostic purposes. Walzer,[10, 12] Gay and Chant,[13] Sherman and Stull,[14] and other early investigators observed that allergen migrated from an injected sensitized site to uninjected sites if too many sites were injected or if challenging solutions which were too concentrated were used. More recently, Richter, Harter, Sehon, and Rose,[15] in studying the allergenic relationships of fractions isolated from ragweed pollen, encountered difficulty owing to migration of allergen in multiple-site testing.

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They also experienced difficulty in distinguishing nonspecific reactions when repeated, direct injections into sensitized sites were used to test for reagin neutralization. Attempts to eliminate or minimize the effect of migration of allergen have been made by reading reactions within five to twenty minutes after challenge and by using quantities of allergen close to the maximal combining capacity of reagins in the sites. Quantitative determination of reagin neutralization is affected more by migration than is the determination of reaction-inciting capacity because reagin neutralization can take place with a quantity of allergen too small to produce a visible reaction in an adjacent site. Control tests containing allergen also complicate the problem of migration in quantitative tests. The quantitative measurement of migration of intracutaneously injected CS-13 in passive transfer tests will be reported in another article.

The principal objections to comparison of allergenic fractions by multiple-site testing on a recipient and challenging by injection of allergen directly into the sensitized sites are as follows: (1) the recipient is caused considerable discomfort by the many sensitizations, challenges and control injections, (2) trauma of sensitized sites by direct injection, particularly where done repeatedly, makes difficult the distinction of specific from nonspecific reactions, and (3) interference caused by migration of allergen from sites injected with quantities of allergen in excess of that specifically bound by reagins. These objections are eliminated in the method described in this article.

The magnitude of the effect of migration on quantitative determinations of allergens may vary from negligible to significant with different systems studied and with different conditions of testing. In critical research studies, however, complete elimination of any possible influence of migration is essential. In the present method, the effect of migration was eliminated by using one test site on each recipient. The effect of trauma due to direct injection of sites and the need for control tests were eliminated by challenging sites by subcutaneous injection of allergen elsewhere in the body.[13, 14, 16-19] Feinberg and Bernstein[18] attempted to eliminate these objections by sensitizing with a series of twofold serial dilutions of the serum on a single recipient and challenging these sites by injection of allergen elsewhere in the body. Challenging of sites by subcutaneous administration of allergen required elimination of the excess allergen from the system before re-use of the recipient. The time interval that injected or injested allergen remains in the system has been determined by previous investigators. Levine and Coca[20] demonstrated timothy pollen allergen in the blood forty-eight hours after intravenous injection of a relatively large dose of timothy pollen extract; after seven days, detection was considered positive but questionable. Tuft[21] reported that traces of horse serum antigen were detectable in the blood seventeen days after intramuscular or intraperitoneal injection of 50 ml. of horse serum. Cohen, Ecker, and Rudolph[22] detected ragweed allergen in the blood twenty-four hours after blowing the pollen into the nose, but the

allergen was not detectable in eight of ten tests forty-eight and seventy-two hours later. Walzer and Walzer[23] observed that peanut allergen was eliminated from the blood forty-eight hours after ingestion of peanuts. It appears that time of retention of allergen in the body varies and depends on the amount given and the nature and probably the molecular size of the allergen. Ninety-six hours was allowed for the elimination of cottonseed allergen after subcutaneous injection.

ABBREVIATIONS USED

The symbols "γ," as used in the tables, and "mcg.," as used in the text, both refer to "microgram" (0.000,001 gram).

MATERIAL USED

CS-1A.—Cottonseed allergen, CS-1A, was isolated from depigmented, defatted cottonseed, as previously described.[5, 6]

CS-13.—Fraction CS-13 was prepared from CS-1A by precipitation with picric acid and recovery of active fraction by removal of the picric acid as described before.[7] Seventy-eight grams of CS-13, containing 17.0 per cent nitrogen (air-dried basis), was obtained from 235 grams of CS-1A.

Dialysis and Ion-Exchange Fractionation of CS-13.—The fractions obtained by ion-exchange fractionation of the dialysate (D series) and endo (E series) fractions of CS-13 have been described in detail.[9] Properties of fractions pertinent to this article are given in Table I. Demonstration by chemical and immunologic methods that (CS-13D)1A and (CS-13E)5F6 were free of each other has been described.[9]

TABLE I. COMPOSITION AND PROPERTIES OF PERTINENT COTTONSEED ALLERGENIC FRACTIONS

FRACTION	NITROGEN* (PER CENT)	CARBOHYDRATE* (PER CENT)	LOWEST CONCENTRATION OF FRACTION N GIVING PRECIPITIN REACTION† (γ/ML.)	MOLECULAR WEIGHT	REFERENCE
CS-1A	13.7‡	—			5-6
CS-13	17.0‡	—			7
(CS-13D)1A	17.5	3.1	None at 200	5 to 12 × 10^3	9
(CS-13E)1A	10.4	26.2	0.7	5 to 12 × 10^3	9
(CS-13E)5F6	20.1	0.45§	0.17	10 to 18 × 10^3	9
CS-13D	19.4	0.6	0.35		9
CS-13E	18.6	6.1	0.35		9

*Ash-water free basis except as noted.
†Cottonseed allergen rabbit antiserum was used.[9]
‡Air-dried basis.
§Estimated from nitrogen content.[26]

Reaginic Blood Serum.—A sufficient quantity of blood serum was collected from a cottonseed-sensitive subject (L.C.), so that all passive transfer tests were done with one lot. The serum was placed in sterile 15 ml. bottles, frozen, and

stored at –20° C. Serum was thawed and reserved at 5° C. at least one week before use. Once thawed, the serum was not refrozen since repeated freezing and thawing rapidly decreased its reagin content.

Test Subjects.—Recipients for passive transfer tests were volunteers at the District of Columbia Workhouse, Occoquan, Virginia,* who gave a negative skin test with CS-1A. Most of the recipients had a negative history of allergy. Cottonseed was eliminated from the diet, and recipients did not receive antihistaminic drugs during the tests.

Test Solutions.—Allergen fractions were dissolved in physiologic salt solution. Stock solutions were sterilized by heating at 100° C. for twenty minutes on three successive days. Dilutions of stock solutions were prepared aseptically with physiologic salt solution.

EXPERIMENTAL METHOD FOR COMPARING THE POTENCY AND CROSS-NEUTRALIZING CAPACITY OF TWO ALLERGENIC FRACTIONS BY PASSIVE TRANSFER

General Consideration.—A reaction which gave a discernible wheal in *exactly* sixty minutes was considered positive. The longest dimension of the wheal was measured and reactions were designated as follows: ±, questionable wheal; 1+, wheal up to 6 mm.; 2+, wheal from 7 to 12 mm.; 3+, wheal from 13 to 20 mm.; 4+, wheal over 20 mm.

The sensitivity of the skin varies significantly on different areas of the arms. Accordingly, sensitization was carried out in the same relative spot on opposite arms for the two phases of the test. Four pairs of sites were selected, two on the top of the biceps and two on the forearms. Designation and location of the sites were as follows: sites 1 and 2 were 87 and 37 mm., respectively, below the elbow bend on the anterior aspect of the forearm; sites 3 and 4 were 65 and 115 mm., respectively, above the elbow bend along the top of the biceps. Sites were located precisely with a celluloid T-square with 6 mm. holes appropriately spaced from the top of edge of the T-square which was laid on the outstretched arm with the elbow bend as reference point.

The interval required for elimination of CS-13 from the system after subcutaneous challenge was determined as follows: Eleven recipients were sensitized on the right arm, site 3, with 0.05 ml. of serum. Sites were challenged twenty-four hours later by subcutaneous injection of 32 mcg. of CS-13 nitrogen in the left arm. Twenty-four hours after this challenge, recipients were sensitized on the left arm, site 3, and challenged twenty-seven hours later by subcutaneous injection of 32 mcg. of CS-13 nitrogen in the right arm. Ninety-six hours after this challenge, recipients were again sensitized on the right arm, site 4, and challenged twenty-one hours later by subcutaneous injection of 32 mcg. of CS-13 nitrogen in the left arm. Readings were taken thirty and sixty minutes after each challenge. Results are shown in Table II. With the thirty-minute reading, the reactions were smaller in some

*The authors wish to express their appreciation to Donald Clemmer, Director of the Department of Corrections, and William F. Fleming, Superintendent, District of Columbia Workhouse, for their kind cooperation in permitting inmates to volunteer for these studies.

TABLE II. ELIMINATION OF CS-13 AFTER SUBCUTANEOUS INJECTION

RECIPIENT	TIME OF SENSITIZATION IN HOURS AFTER PRECEDING CHALLENGE*					
	30-MINUTE READING			60-MINUTE READING		
	ORIGINAL	24	96	ORIGINAL	24	96
B. C.	3+	3+	3+	3+	3+	4+
R. E.	2+	0	2+	2+	3+	3+
W. M.	3+	2+	3+	3+	3+	4+
M. P.	2+	0	2+	3+	3+	3+
W. B.	0	±	0	4+	3+	3+
W. W.	2+	3+	2+	3+	3+	4+
J. R.	4+	3+	3+	4+	4+	4+
J. L.	2+	±	2+	3+	2+	3+
R. C.	2+	0	3+	4+	4+	4+
F. C.	3+	0	3+	3+	3+	3+
C. T.	3+	3+	3+	3+	3+	3+

*Each challenge was made with 32 mcg. of CS-13 nitrogen administered subcutaneously.

cases with the twenty-four-hour interval after the first challenge with CS-13 than they were with the original test or with the ninety-six-hour interval test. With the sixty-minute reading, however, the reaction size was equal with the original and the twenty-four- and ninety-six-hour tests. These results showed that if allergen were retained for twenty-four hours after challenge, as indicated by the slightly lower thirty-minute readings, it was in extremely small amount.* To ensure that allergen was eliminated completely, a ninety-six-hour interval was allowed before continuing the second part of the experiment described below.

The method for comparing the passive-transfer-reaction-inciting, reagin-neutralization, and cross-neutralization capacities of two allergenic fractions is described with (CS-13D)1A and (CS-13E)5F6, as an example. Two groups (I and II) of eleven men each were used for the experiment which required eight meetings during eleven days. In order to eliminate, in so far as possible, the effects of individual variation in reactivity of recipients, both fractions were tested in equal quantities on each recipient in consecutive weeks. Data are shown in Table III, in which results of tests on both groups are shown in each column.

The method as described on a day-by-day basis follows:

First Day, Senitization.—Each recipient in Groups I and II was sensitized in site 3 on the right arm by intracutaneous injection of 0.05 ml. of L.C. serum.

Second Day (Twenty to Twenty-Four Hours After Sensitization) Reaction-Inciting Capacities.—

Group I: One milliliter of a solution containing one of a series of twofold serial dilutions of (CS-13D)1A—0.125 to 32 mcg. of (CS-13D)1A nitrogen per milliliter—was injected subcutaneously into the outer aspect of the upper left arm of each recipient, except for the control. Results are recorded in column *4*.

*Results of a similar unpublished experiment using 136 mcg. of nitrogen of the castor bean allergen, CB-1C, for challenging showed marked retention of allergen for twenty-four hours but complete elimination after ninety-six hours.

TABLE III. COMPARISON OF (CS-13D)1A AND (CS-13E)5F6 BY QUANTITATIVE PASSIVE TRANSFER ANALYSIS

RECIPIENT	GROUP NO.	NITROGEN* (γ/ML.)	(CS-13D)1A RESULT	(CS-13D)1A RESULT† 32 γN/ML.	(CS-13E)5F6 RESULT‡ 32 γN/ML.	(CS-13E)5F6 RESULT	(CS-13E)5F6 RESULT† 32 γN/ML.	(CS-13D)1A RESULT§ 32 γN/ML.
1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
M. H.	I	0.125	0	3+	3+	0	4+	0
W. B.	II		0	3+	3+	0	3+	0
F. T.	I	0.25	0	3+	2+	0	3+	0
R. B.	II		0	0	2+	0	3+	0
P. C.	I	0.50	0	0	0	0	2+	0
G. H.	II		0	0	3+	0	3+	0
R. S.	I	1.0	0	0	3+	0	-‖	-‖
N. N.	II		0	0	3+	±	0	0
L. F.	I	2.0	0	0	3+	±	0	0
D. Q.	II		0	0	0	0	0	0
P. M.	I	4.0	0	0	2+	0	0	0
E. P.	II		0	0	+	3+	0	0
P. P.	I	8.0	±	0	3+	3+	0	0
B. W.	II		3+	0	3+	4+	0	0
C. S.	I	16	3+	0	2+	3+	0	0
J. B.	II		3+	0	3+	3+	0	0
H. R.	I	32	3+	0	3+	3+	0	0
J. M.	II		3+	0	3+	4+	0	0
A. C.	I	64	3+	0	3+	4+	0	0
L. H.	II		3+	0	0	4+	0	0

*(CS-13D)1A and (CS-13E)5F6 nitrogen used for determination of passive transfer inciting capacities as shown by results in columns *4* and *7*, respectively.

†Homologous neutralization by (CS-13D)1A and (CS-13E)5F6 nitrogen used in columns *4* and *7* to 32 mcg. of respective fraction. Results are shown in columns *5* and *8*, respectively.

‡Further reaction caused by 32 mcg. of (CS-13E)5F6 nitrogen in sites neutralized by (CS-13D)1A.

§No further reaction caused by 32 mcg. of (CS-13D)1A nitrogen in sites neutralized by (CS-13E)5F6 is shown in column *9*.

‖Not tested.

Group II: One milliliter of solution containing one of a series of twofold serial dilutions of (CS-13E)5F6—0.125 to 32 mcg. of (CS-13E)5F6 nitrogen per milliliter—was injected similarly into Group II. Results are recorded in column *7*.

Third Day, Neutralization.—

Group I: Each recipient was injected subcutaneously in the outer aspect of the upper left arm with 1 ml. of solution containing 32 mcg. of (CS-13D)1A nitrogen per milliliter. Results are recorded in column *5*.

Group II: Each recipient was similarly injected with 1 ml. of solution containing 32 mcg. of (CS-13E)5F6 nitrogen per milliliter. Results are recorded in column *8*.

Fourth Day, Cross-Neutralization.—

Group I: Each recipient was injected subcutaneously in the outer aspect of the upper left arm with 1 ml. of solution containing 32 mcg. of (CS-13E)5F6 nitrogen per milliliter. Results are recorded in column *6*.

Group II: Each recipient was injected similarly with 1 ml. of solution containing 32 mcg. of (CS-13D)1A nitrogen per milliliter. Results are recorded in column *9*.

Controls: The two recipients, who had not received any allergen since sensitization, were injected subcutaneously on the left arm, one with 1 ml. of (CS-13D)1A and one with 1 ml. of (CS-13E)5F6, containing 32 mcg. of fraction nitrogen, respectively. Control tests were always positive, which showed that the sites remained sensitive during the test interval.

Fifth, Sixth, and Seventh Days, Elimination of Allergen.—A four-day interval was allowed for elimination of allergen from the system before continuing the second part of the experiment.

Eighth Day, Sensitization.—All recipients in Groups I and II were sensitized with 0.05 ml. of L.C. serum in site 3 on the left arm (opposite arm from that used on the first day).

Ninth, Tenth, and Eleventh Days.—The procedure described for the second, third, and fourth days was repeated on the ninth, tenth, and eleventh days, respectively, except that the allergen fraction used on each group was reversed. For example, (CS-13E)5F6 was used on Group I and (CS-13D)1A was used on Group II on the ninth day and so on. Results are shown in appropriate columns of Table III.

RESULTS AND DISCUSSION

The reproducibility of the method of comparing passive-transfer-inciting and reagin-neutralizing capacities of allergens is shown in Table IV, where results of duplicate tests on four recipients at twofold concentration levels ranging from 0.125 to 32 mcg. of CS-13 nitrogen are given. The duplicate tests were made in consecutive weeks with a four-day interval between the end of one test and the start of the next. It is apparent in both incitation and neutralization that results of duplicate tests on the same subject and on different subjects with the same quantities of allergen are in excellent agreement. In incitation of reaction, ten of fifteen duplicates agreed perfectly, five varied by 1+ unit, and one varied by 2+ units. This latter test, V.A. at the 2 mcg. level, was the only one in which a positive reaction was obtained in one test and a negative reaction in a duplicate test.

No positive reaction was obtained with less than 2 mcg. of CS-13 nitrogen. One positive reaction was obtained with 2 mcg., and all reactons were positive with all levels from 4 to 32 mcg. of CS-13 nitrogen with all recipients, except for one ± reaction. In neutralization of reagins, nine of twelve duplicates agreed perfectly, two differed by 1+, and one differed by 2+ units. Agreement of results among the four individuals tested at each level was excellent. All sites were neutralized by 1 mcg. of CS-13 nitrogen, with three ± reactions at the 1 mcg. level. The reproducibility of the method in determination of both the passive-transfer-inciting and reagin-neutralizing capacities was

TABLE IV. PRECISION OF QUANTITATIVE METHOD FOR PASSIVE TRANSFER ANALYSIS WITH CS-13

RECIPIENT	INCITATION OF REACTION* CS-13 NITROGEN (γ/ML.)	RESULT I†	RESULT II‡	NEUTRALIZATION OF REAGINS§ CS-13 NITROGEN (32 γ/ML.) RESULT I	RESULT II
J. L.	0.125	0	0	4+	3+
P. M.		0	0	4+	4+
L. V.		0	0	4+	4+
R. P.		0	0	4+	2+
H. H.	0.25	0	0	3+	3+
W. S.		0	0	4+	4+
J. O.		0	0	4+	4+
J. B.		0	0	4+	4+
W. K.	0.50	0	0	3+	3+
J. A.		0	0	2+	2+
J. R.		0	0	4+	3+
O. G.		0	0	4+	4+
M. H.	1.0	0	0	0	±
A. B.		0	0	0	0
J. L.		0	0	±	0
P. M.		0	0	±	0
W. N.	2.0	0	0	0	0
V. A		2+	0	0	0
L. F.		0	0	0	0
W. S.		0	0	0	0
C. H.	4.0	1+	±	0	0
W. W.		3+	4+	0	0
W. K.		3+	3+	0	-‖
J. A.		3+	3+	0	0
J. R.	8.0	3+	4+	0	0
O. G.		3+	4+	0	0
W. H.		0	-‖	0	-‖
V. A.		4+	4+	0	0
J. O.	16	4+	4+	0	0
J. B.		4+	4+	0	0
M. H.		3+	3+	0	0
A. B.		4+	4+	-‖	0
L. V.	32	3+	3+	0	0
R. P.		3+	4+	0	0
C. H.		4+	4+	0	0
W. W.		4+	4+	0	0

*Sites challenged by subcutaneous injection of 1 ml. of solution containing indicated quantity of CS-13 nitrogen per milliliter in outer aspect of upper arm opposite sensitized sites. Challenge was twenty-four hours after sensitization.

†Sensitization in site 3, right arm.

‡Sensitization in site 3, left arm, ninety-six hours after last challenge injection.

§Sites challenged by subcutaneous injection of 1 ml. of solution containing 32 mcg. CS-13 nitrogen per milliliter into outer aspect of upper arm opposite sensitized site. Injection was made twenty-four hours after the challenge test for determination of incitation of reaction.

‖Not tested.

judged to be such that serial dilutions, differing by only twofold, could be distinguished.

Comparisons of the passive-transfer-reaction-inciting, reagin-neutralizing, and cross-neutralizing capacities of (CS-13D)1A and (CS-13E)5F6 are shown in Table III. (CS-13E)5F6 was twice as potent as (CS-13D)1A in inciting reactions, because 8 and 16 mcg. of respective fraction nitrogen were required to

incite reaction with both recipients, with one recipient having reacted positively with 4 and 8 mcg. of nitrogen of the respective fractions. In contrast, (CS-13D)1A was twice as potent as (CS-13E)5F6 in homologous reagin neutralization because 0.5 and 1.0 mcg. of respective fraction nitrogen were required to prevent further reaction to 32 mcg. of homologous fraction nitrogen.

(CS-13D)1A did not neutralize sites to subsequent test with (CS-13E)5F6, but (CS-13E)5F6 neutralized to (CS-13D)1A, as shown in columns *6* and *9*, respectively, of Table III. If (CS-13E)5F6 had not neutralized sites to subsequent test with (CS-13D)1A, the evidence that their specificities were different would have been conclusive. However, Clarke,[24] Harkavy and Witebsky,[25] and Sherman and Stull[14] have reported that, with certain sera, unrelated allergens sometimes effected cross-neutralization of reagins. If nonspecific cross-neutralization has occurred in this case, the results would indicate that the specificities of (CS-13D)1A and (CS-13E)5F6 were different. For the present, an alternative interpretation that (CS-13D)1A possessed only part of the specificity determinant grouping found in (CS-13E)5F6 also must be regarded as possible.

Fraction (CS-13D)1A represented the maximum separation from (CS-13E)5F6 involving both dialysis and ion-exchange fractionation, whereas (CS-13E)1A represented maximum separation from (CS-13E)5F6 by ion-exchange fractionation alone.[9] Like (CS-13D)1A, (CS-13E)1A was completely separated from (CS-13E)5F6, as shown by an ion-exchange characterization curve like that in Fig. 4 of Ref. 9. Also, like (CS-13D)1A, (CS-13E)1A did not neutralize to (CS-13E)5F6, but (CS-13E)5F6 neutralized sites to subsequent test with (CS-13E)1A. These results are of interest because (CS-13D)1A gave no precipitin reaction with 200 mcg. per milliliter of (CS-13D)1A nitrogen, whereas (CS-13E)1A and (CS-13E)5F6 gave precipitin reactions with threshold concentrations of 0.7 and 0.17 mcg. of respective fraction nitrogen per milliliter with rabbit CS-13E antiserum.[9] Interpretation of these relationships is complicated because the carbohydrate contents of (CS-13D)1A, (CS-13E)1A, and (CS-13E)5F6 were 3.1, 26.2, and 0.45 per cent, respectively. Whether (CS-13E)1A gave a precipitin reaction because of its higher carbohydrate content than (CS-13D)1A or because the undialyzed (CS-13E)1A represented a larger protein molecule per se is not known. (CS-13E)1A was one-fourth as potent as (CS-13E)5F6 in reaction-inciting capacity and equally as potent as (CS-13E)5F6 in homologous reagin neutralization.

The potencies of (CS-13E)5F6, CS-13, and CS-1A were compared to ascertain whether the many steps involved in the preparation of CS-13 and (CS-13E)5F6 from CS-1A had significantly lessened the activity of these fractions. This was done by comparing both CS-1A and (CS-13E)5F6 to CS-13. CS-13 and CS-1A (Table V) were equally effective in inciting passive transfer reactions, as 4 mcg. of nitrogen of each fraction was required to cause reaction in both subjects (in both cases one positive reaction was produced by 2 mcg. of fraction nitrogen) as shown in columns *4* and *7*, respectively. In neutralization, however, CS-13 was four times more effective than CS-1A, because 0.5

TABLE V. COMPARISON OF CS-1A AND CS-13 BY QUANTITATIVE PASSIVE TRANSFER ANALYSIS

RECIPIENT	GROUP NO.	FRACTION NITROGEN (γ/ML.)*	CS-13		CS-1A	CS-1A		CS-13
			RESULT	RESULT† 32γN/ML.	32 γN/ML.‡	RESULT	RESULT† 32γN/ML.	32 γN/ML.‡
1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
D. McN.	I	0.125	0	4+	0	0	4+	0
J. W.	II		0	4+	0	0	4+	0
A. E.	I	0.25	0	3+	0	0	4+	0
J. D.	II		0	3+	0	0	4+	0
D. McG.	I	0.50	0	0	0	0	0	0
H. D.	II		0	0	0	0	2+	0
S. P.	I	1.0	0	0	0	0	3+	0
E. A.	II		0	0	0	0	2+	0
D. A.	I	2.0	2+	0	0	0	0	0
F. S.	II		0	0	0	3+	0	0
A. D.	I	4.0	3+	0	0	3+	0	0
D. L.	II		3+	0	0	2+	0	0
A. C.	I	8.0	3+	0	0	4+	0	0
P. Y.	II		3+	0	0	3+	0	0
T. M.	I	16	4+	0	0	4+	0	0
L. F.	II		4+	0	0	3+	0	0
N. B.	I	32	3+	0	0	4+	0	0
G. R.	II		3+	0	0	3+	0	0

*Nitrogen of respective fractions used in columns *4* and *7* to determine passive-transfer-inciting capacities.

†Homologous neutralization with 32 mcg. of nitrogen of respective fractions.

‡Cross-neutralization.

and 2.0 mcg. of respective fraction nitrogen were required to neutralize the sites (columns *5* and *8*). CS-13 and (CS-13E)5F6 (Table VI) were essentially equal in inciting reactions, as CS-13 gave one positive with 1 and 2 mcg. of nitrogen, and (CS-13E)5F6 gave one positive at 1 mcg. and both positives with 2 mcg. as shown in columns *4* and *7*, respectively. Recipients H. D. and D. McG. were refractory. Both SC-13 and (CS-13E)5F6 were equally potent in reagin neutralization, as both required 1 mcg. of nitrogen to neutralize sites (columns *5* and *8*).

(CS-13E)5F6 represents 1.25 and 2.74 per cent of precursor fractions CS-1A and CS-13, respectively, on a nitrogen basis. Yet, the passive-transfer-inciting capacities of the three fractions were equal and the reagin-neutralizing capacities of (CS-13E)5F6 and of CS-13 were only four times as great as that of CS-1A. This observation substantiates our previous opinion that, essentially, CS-1A and CS-13 represent a mixture of closely related chemical components possessing a common allergenic specificity, because sites neutralized to highly purified (CS-13E)5F6 were also neutralized to CS-13 and CS-1A. Excluded from this consideration, however, are the possible different specificities of (CS-13D)1A and (CS-13E)1A which represent relatively minor fractions of CS-13. Immediate precursor fractions of (CS-13D)1A and (CS-13E)1A, (CS-13D)1 and (CS-13E)1, respectively, neutralized

TABLE VI. COMPARISON OF CS-13 AND (CS-13E)5F6 BY QUANTITATIVE PASSIVE TRANSFER ANALYSIS

RECIPIENT	GROUP NO.	FRACTION NITROGEN (γ/ML.)*	CS-13		(CS-13E)5F6	(CS-13E)5F6		CS-13
			RESULT	RESULT† 32γN/ML.	32 γN/ML.‡	RESULT	RESULT† 32γN/ML.	32 γN/ML.‡
1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
D. McN.	I	0.125	0	4+	0	0	3+	0
J. W.	II		0	4+	0	0	4+	0
A. E.	I	0.25	0	3+	0	0	4+	0
L. F.	II		0	3+	0	0	3+	0
N. B.	I	0.50	0	3+	0	0	3+	0
G. R.	II		0	3+	0	0	3+	0
A. C.	I	1.0	1+	±	0	2+	0	0
D. L.	II		0	0	0	±	0	0
A. D.	I	2.0	2+	0	0	3+	0	0
J. D.	II		0	0	0	2+	0	0
T. M.	I	4.0	3+	0	0	3+	0	0
J. M.	II		4+	0	0	4+	0	0
L. B.	I	8.0	3+	0	0	3+	0	0
H. D.	II		0	0	0	0	0	0
D. A.	I	16	3+	0	0	3+	0	0
F. S.	II		3+	0	0	2+	0	0
D. McG.	I	32	0	0	0	0	0	0
D. R.	II		4+	0	0	4+	0	0

*Nitrogen of respective fractions used in columns 4 and 7 to determine passive-transfer-inciting capacities.

†Homologous neutralization with 32 mcg. of nitrogen of respective fractions.

‡Cross-neutralization.

sites to further test with (CS-13E)5F6 and (CS-13D)1 and (CS-13E)1 represented less than 10 per cent of CS-13. Fraction (CS-13E)5F6 appears to be approaching homogeniety because (1) it gave only one line of precipitate by the Ouchterlony method,[9] (2) it gave a precipitin reaction with the highest dilution, on a nitrogen basis, of any of the fractions,[9] (3) it is essentially carbohydrate-free as estimated from its nitrogen content, and (4) it sedimented uniformly in the ultracentrifuge.[9] Other criteria would be required for conclusive evidence of homogeniety.

SUMMARY

A quantitative method for comparing three properties of two allergenic fractions by the passive transfer technique is described. The properties are: (1) the passive-transfer-reaction-inciting capacities, (2) the reagin-neutralizing capacities, and (3) whether or not the specificities of the fractions are the same. The reproducibility of the method in the determination of both passive-transfer-inciting and reagin-neutralizing capacities was such that serial dilutions differing only twofold could be distinguished. The method eliminates the interfering effect of migration of allergen and the need for control tests. The method was demonstrated with subfractions, (CS-13D)1A

and (CS-13E)5F6, of the cottonseed allergen, CS-1A, which were completely free of each other. The significance of the observations made in evaluation of the separated allergenic components of CS-1A is discussed.

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QUANTITATIVE MEASUREMENT OF THE MIGRATION OF INTRACUTANEOUSLY INJECTED COTTONSEED ALLERGEN IN PASSIVE TRANSFER STUDIES

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INTRODUCTION

THE passive transfer technique is frequently used to evaluate allergenic properties of fractions isolated from natural sources or of modified preparations. Multiple sensitized site testing on the arms or back of a recipient usually has been used for these studies. Migration of allergen in excess of that combinable with reagins in the injected site and its possible invalidation of results has been recognized,[1] but quantitative measurements of migration with a well-defined allergen have not been described.

Need for a critical method of comparing the passive-transfer-inciting capacities, the reagin-neutralization capacities, and cross-neutralization relationships of isolated cottonseed allergenic fractions prompted development of a method which completely eliminated the problem of interference by migration. This method is described in our preceding article.[2] The literature on this subject was reviewed in that article and need not be repeated here.[2]

The object of this article is to describe the quantitative determination of migration of the cottonseed allergen, CS-13, in inciting passive transfer reactions and in neutralizing reagins in uninjected sensitized sites adjacent to challenged sites.

ABBREVIATIONS USED

The symbol "mcg." refers to "microgram" (0.000,001 gram). The symbol "Mγ," used in the tables, refers to "millimicrogram" (0.000,000,001 gram).

MATERIAL USED

Fraction CS-13, test solutions, test subjects, and the LC cottonseed reaginic serum have been described.[2] The CN cottonseed reaginic serum was stored at 5° C.

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EXPERIMENTAL

Sensitization.—Each of twelve recipients was sensitized intracutaneously with 0.05 ml. of serum in two sites 50 mm. apart. Choice of site location is discussed in the preceding article.[2] For the LC serum test, the top of the left biceps was used; sites 3 and 4 were 65 and 115 mm. above the elbow bend, respectively. For the CN serum test, the anterior aspect of the right forearm was used; sites 1 and 2 were 87 and 37 mm. below the elbow bend, respectively.

TABLE I. INCITATION OF PASSIVE TRANSFER REACTION BY MIGRATION OF ALLERGEN

RECIPIENT	SITE*	CS-13 NITROGEN (MγN)	LC SERUM†		CN SERUM‡	
			30 MIN.	60 MIN.	30 MIN.	60 MIN.
J. H.	I	1	2+	2+	2+	2+
	U	0	0	0	0	0
H. M.	I	2	2+	2+	2+	2+
	U	0	0	0	0	0
A. H.	I	4	3+	3+	2+	2+
	U	0	0	0	0	0
G. W.	I	8	2+	2+	3+	3+
	U	0	0	0	0	0
J. C.	I	16	3+	3+	3+	3+
	U	0	0	0	0	0
J. Ca.	I	32	2+	3+	2+	3+
	U	0	0	0	0	0
J. M.	I	64	2+	3+	3+	3+
	U	0	0	0	0	0
P. P.	I	128	3+	4+	4+	4+
	U	0	0	0	0	0
W. W.	I	256	3+	2+	3+	4+
	U	0	0	0	0	0
J. D.	I	512	4+	4+	3+	3+
	U	0	0	0	0	0
R. B.	I	1,024	3+	3+	4+	4+
	U	0	0	2+	0	0
H. T.	I	2,048	4+	4+	4+	4+
	U	0	±	4+	0	3+

*I = Injected site; U = uninjected site.
†Sites 3 and 4 were used, site 3 being the injected site.
‡Sites 1 and 2 were used, site 1 being the injected site.

Four days after completion of the test with the LC serum, the same recipients were sensitized for a similar test with CN serum. This interval between tests ensured elimination of excess allergen from the system from the previous test.[2]

Measurement of Reaction.—A reaction which gave a discernible wheal was considered positive. Reactions were measured, using the longest dimension of the wheal, and designated as follows: ±, questionable wheal; 1+, wheal up to 6 mm.; 2+, wheal from 7 to 12 mm.; 3+, wheal from 13 to 20 mm.; 4+, wheal over 20 mm.

Determination of the Effect of Migration of Allergen in Inciting Passive Transfer Reactions.—Twenty-four hours after sensitization, 0.025 ml. of CS-13 solution was injected intracutaneously into sites 1 and 3 for the CN and LC sera tests, respectively. The concentrations were adjusted so that the first recipient received 1 millimicrogram of CS-13 nitrogen and each subsequent recipient received a twofold increase in CS-13 nitrogen, the range being from 1 to 2,048 millimicrograms per test site. The size of the wheals in the injected and the uninjected sites was recorded thirty and sixty minutes after challenge.

TABLE II. NEUTRALIZATION OF REAGINS IN PASSIVELY SENSITIZED SITES BY MIGRATION OF ALLERGENS

RECIPIENT	SITE*	CS-13 NITROGEN (Mγ)	REACTION IN 60 MINUTES†	
			LC SERUM	CN SERUM
J. H.	I	1	3+	3+
	U	0	4+	4+
H. M.	I	2	0	2+
	U	0	3+	4+
A. H.	I	4	0	0
	U	0	0	0
G. W.	I	8	0	0
	U	0	3+	4+
J. C.	I	16	0	0
	U	0	3+	3+
J. Ca.	I	32	0	0
	U	0	±	3+
J. M.	I	64	0	0
	U	0	3+	0
P. P.	I	128	0	0
	U	0	0	0
W. W.	I	256	0	0
	U	0	2+	0
J. D.	I	512	0	0
	U	0	0	0
R. B.	I	1,024	0	0
	U	0	0	0
H. T.	I	2,048	0	0
	U	0	0	0

*I refers to the site injected twenty-four hours earlier with the amount of CS-13 nitrogen shown under the next column to the right. U refers to the uninjected site. Results of this test were shown in Table I.

†Reaction sixty minutes after subcutaneous injection of 32 mcg. of CS-13 nitrogen into arm opposite that having the sensitized sites. Both sites I and U were negative when this challenge injection was made.

Control tests on the recipients by intracutaneous injection of 0.025 ml. of solution containing from 1 to 512 millimicrograms of CS-13 nitrogen were negative six days before the migration test. Results are shown in Table I.

Determination of the Effect of Migration of Allergen in Neutralizing Reagins in Passively Sensitized Sites.—Test for neutralization of reagins in both the injected and uninjected sites was made twenty-four hours after the intracutaneous challenge. One milliliter of solution containing 32 mcg. of CS-13

nitrogen was injected subcutaneously into the outer aspect of the upper arm opposite to that which had the sensitized sites. The size of the wheal was measured in both sites sixty minutes after challenge. Previous tests had shown that 32 mcg. of CS-13 nitrogen injected in this way was eight times greater than the threshold amount required to produce positive reactions in passively sensitized sites.[2] Control tests were not needed with this method of challenge.

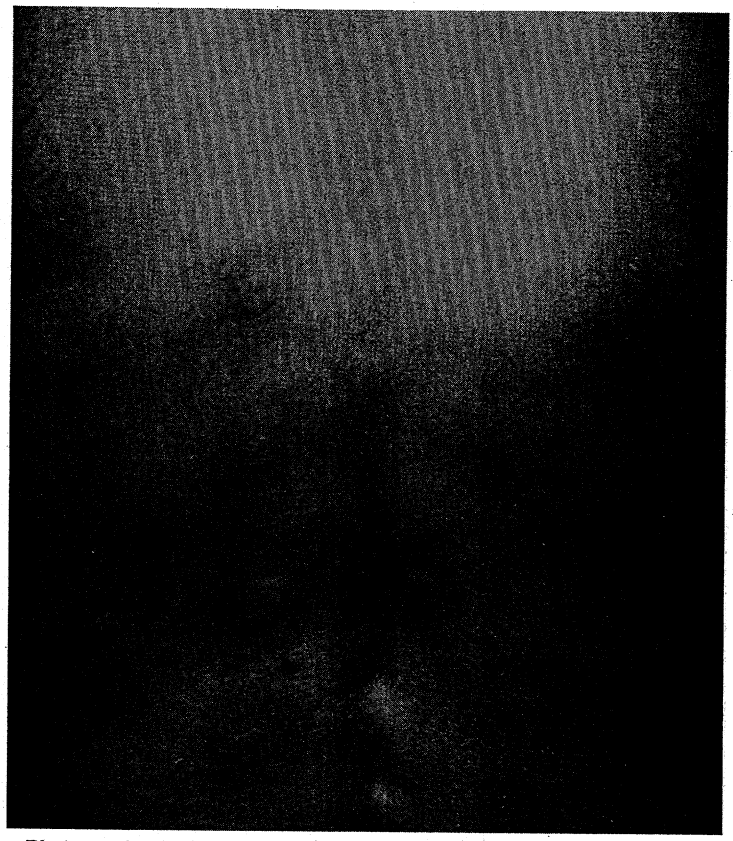

Fig. 1.—Photograph showing migration of CS-13 on back. Two mcg. of CS-13 nitrogen was injected intracutaneously into site designated with arrow thirty-two minutes before picture was taken. Surrounding, uninjected sites 50 mm. distant and 100 and 200 mm. above injected site reacted by migration of CS-13.

RESULTS AND DISCUSSION

The amount of CS-13 nitrogen required to incite positive passive transfer reactions and to neutralize reagins by migration of allergen from an injected site to an uninjected site 50 mm. distant was determined by testing with twofold serial dilutions over the range of 1 to 2,048 millimicrograms. Results are shown in Tables I and II. Reaction was incited in injected sites with both sera with all concentrations of allergen. The threshold amounts required to incite wheals by migration to uninjected sites were 1,024 and 2,048 millimicrograms of CS-13 nitrogen for the LC and CN sera, respectively. Redness, but no wheals, was

produced in a few uninjected sites with lesser amounts of CS-13 nitrogen. The threshold amounts of CS-13 nitrogen required to neutralize reagins by migration from the injected to the uninjected site, 50 mm. distant, were 512 and 64 millimicrograms with LC and CN sera, respectively. In contrast, neutralization of reagins in the injected sites was effected by 2 and 4 millimicrograms of CS-13 nitrogen with the LC and CN sera, respectively.

These results show that over 1,000 times as much allergen was required to incite reaction by migration to the uninjected site as was required to incite reaction by direct injection, but that only sixteen to 100 times as much allergen was required to neutralize the reagins by migration to the uninjected site as was required to neutralize reagins in the injected site. It is apparent that reagin neutralization can occur in uninjected sites by migration of allergen without any visible reaction.

The mechanism of migration of intracutaneously injected allergen to adjacent uninjected sites probably involves simple diffusion, as well as transport by lymph and blood circulatory systems. To determine which of these factors predominated, three sites, 50 mm. apart, were sensitized on the upper aspect of the biceps on the right arm and one site was sensitized on site 2, left forearm of a recipient, with 0.05 ml. CN serum injected in each site. Twenty-four hours after sensitization, the middle site of the group of three was injected intracutaneously with 0.025 ml. of solution containing 2,048 millimicrograms of CS-13 nitrogen. Sixty minutes after this challenge, wheals had formed as follows: right arm, lower site, 3+; middle (injected) site, 4+; upper site, 3+; site on left arm, negative. This indicated that migration for short distances involved diffusion primarily, for if circulatory transport were involved the site on the left arm also should have reacted. Circulatory transport undoubtedly predominates when allergen is absorbed. In another experiment, allergen was shown to migrate from a site on the biceps injected with 0.025 ml. of solution containing 1,024 millimicrograms of CS-13 nitrogen to give 3+ and 2+ reactions in sixty minutes, in sites 2 and 1, respectively, on the same forearm. This indicated that allergen migrated against the direction of lymph circulation. Fig. 1 shows a pattern of migration on the back, where movement of allergen was possibly more rapid than on the arms. Whereas uninjected sites were negative on the arms after thirty minutes, the sites on the back became red in fifteen minutes, with the wheals, as shown in Fig. 1, appearing in thirty minutes. The site marked with the arrow had been injected with 0.025 ml. of solution containing 2,048 millimicrograms of CS-13 nitrogen thirty-two minutes before the photograph was taken. The four sites surrounding the injected site are 50 mm. distant, while the sites above these are 100 and 200 mm. from the injected site. It is evident that migration occurred laterally as well as above and below the injected site.

The rate of migration of intracutaneously injected allergens probably varies, depending on their molecular weights. CS-13 may migrate faster than other allergens because the molecular weights of subfractions of CS-13 fractions ranged from 5,000 to 18,000.[2]

SUMMARY

Quantitative measurement of the migration of the cottonseed allergen, CS-13, in inciting positive passive transfer reactions and in neutralizing reagins by migration to an uninjected site 50 mm. from the injected site is described. Implications of these observations on interpretation of results obtained in comparing allergen fractions by multiple sensitized site testing on a single recipient are discussed. The results justify development of the method of evaluating allergens (described in our preceding article) which eliminates interference by migration.

REFERENCES

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