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STUDIES ON THE HOUSE DUST ALLERGEN(S)

I. THE RELATIONS BETWEEN ANTIGENIC AND ALLERGENIC COMPONENTS

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THE source of the skin-reactive components present in extracts made from house dust, cotton, or kapok, especially after aging, has engaged the attention of many workers since house dust was first described by Cooke¹ as one cause of symptoms in patients with rhinitis and asthma. The object of the experiments reported herein was to identify in such materials those components inducing complement-fixing and precipitating antibody in rabbits and to study the relation between these and that giving rise to skin reactions in clinically dust-sensitive persons. If successful, such a step might facilitate later attempts to trace the source of such antigenic and allergenic materials as are present in them.

MATERIALS AND METHODS

Dust Extracts.—Extract E, used as the principal antigen, was a lyophilized light brown powder obtained by precipitation with acetone and dioxane from an aqueous extract of old cotton mattress stuffing.* One gram of the powder was readily soluble in 10 ml. of physiologic saline to give a 1:10 dilution, a somewhat viscid, dark brown solution; clear-cut skin reactions were obtainable in selected persons with dilutions of 1:40,000 or higher.

Extract C was a purified fraction prepared from a house dust extract kindly supplied by Dr. D. H. Campbell.²

Adjuvant Mixture.—Sterile technique was used in preparing the adjuvant mixture, which contained one part of Falba, 1.2 parts of Bayol F containing

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20 mg./100 ml. of heat-killed, dried *Mycobacterium butyricum*, and 1.8 parts of a glycerinated extract E (1 gm./60 ml.). This mixture was freshly prepared each time injections were given.

Antisera.—The schedule followed in immunizing the rabbits is shown in Table I. Two groups were injected with extract E. Positive results in complement fixation tests were observed with the antisera from the first group of five rabbits (Nos. 1 to 5), but the precipitin reactions were weak and the sera were not useful in agar diffusion tests.

TABLE I. IMMUNIZATION OF RABBITS WITH DUST EXTRACT E

DATE	RABBIT NO.	ROUTE	NUMBER OF SITES INJECTED	TOTAL EXTRACT* INJECTED (ML.)	BLEEDING NO.
<i>First Group</i>					
7/31/53	1-5	Subcutaneous	2	1.0†	
9/16	1-5	Subcutaneous	2	1.0†	
10/22					1
11/6	1-5	Intracutaneous	6	1.2-2.0	
12/23	1-5				2
3/16/54	1-5	Subcutaneous	1	1.0 of 1:400	
3/18	1-5		1	1.0 of 1:200	
3/23	1-5				3
<i>Second Group</i>					
11/12/53	6-11	Intracutaneous	10	2.6-3.0**	
11/13	12-15	Intracutaneous	10	3.0-4.0**	
12/23	6, 7, 8, 13, 14, 15				1
12/29	9-12				1
3/16/54	6-14	Subcutaneous	1	1.0 of 1:400	
3/18	6-14	Subcutaneous	1	1.0 of 1:200	
3/19	6-14	Subcutaneous	1	1.0 of 1:120	
3/23	6, 7				2
3/24	8, 9, 10, 13				2
3/25	11, 12, 14				2
4/14	10				3
5/18	14	Subcutaneous	1	1.0 of 1:400	
5/21	14	Subcutaneous	1	1.0 of 1:120	
5/26	14				3
10/20	6-9	Subcutaneous	1	0.2 of 1:400	
10/21	6-9	Subcutaneous	1	0.35 of 1:400	
10/22	6-9	Subcutaneous	1	1.0 of 1:400	
10/25	6-9	Subcutaneous	1	1.0 of 1:400	
10/27	6-9	Subcutaneous	1	1.5 of 1:400	
11/2	6-9				3
11/15	11-13				3
11/23	6-9				4

*In saline solution except where indicated.

†Extract incorporated in adjuvant (see text).

After preliminary bleeding (normal sera) rabbits 6 to 15 were given extract E with adjuvant intracutaneously, followed later by two series of subcutaneous injections of extract diluted 1:120, 1:200, or 1:400 in physiologic saline. The antisera obtained from these rabbits were used satisfactorily in agar diffusion tests.

A total of forty-seven immune bleedings was obtained from the fifteen rabbits, comprising fifteen first bleedings, fourteen second bleedings, fourteen third bleedings, and four fourth bleedings.

Complement Fixation.—The 50 per cent unit of 1:100 guinea pig C' was determined with the Coleman Junior spectrophotometer. Four units and 0.2 ml. of a 1 per cent suspension of washed sheep cells sensitized with 2 units of rabbit antsheep lysin were used in the fixation tests. The fixing and final volumes were 0.3 and 0.5 ml., respectively. The mixture of complement, antiserum, and antigen was incubated for eighteen to twenty-four hours at 4° to 6° C. and again, after the addition of the lytic system, for thirty minutes at 37° C. The diluent for all reagents was Veronal buffer.³ The maximal dose of both antiserum and antigen was one-fourth the anticomplementary dose which, for the antisera, ranged from 0.1 ml. of 1:10 to 0.1 ml. of 1:100; for the dust antigen it was 0.1 ml. of a 1:4,000 dilution. The antigen was usually diluted in twofold steps to 1:64,000 or higher.

Agar Diffusion.—Agar diffusion tests, using 0.6 per cent agar containing 0.4 per cent phenol, were done in tubes by both single diffusion and double diffusion techniques.⁴ With the same concentration of agar, tests were also done in plates,⁵ using Petri dishes 55 to 60 mm. in diameter. Wells were formed with penicillin cups 15 to 30 mm. apart. For comparison of the reactivity of two antisera with one antigen or of two antigens with one antiserum, the three cups were carefully set at the apices of an equilateral triangle. In the majority of the tests with agar in tubes or in plates, the antisera were diluted 1:2, although more dilute or undiluted serum was also used in some instances. The diluent for antiserum and antigen was 0.85 per cent sodium chloride solution containing 0.4 per cent phenol.

RESULTS

Skin Reactions to Extract E in Immunized Rabbits.—The rabbits did not react with either general or local manifestations to the subcutaneous stimulating injections of extract E. Intracutaneous tests, which were read at twenty minutes and again at twenty-four and forty-eight hours, were done at two different dates on some of the rabbits in the second group (Table II). There were no reactions seen at twenty minutes. In the earlier tests, done four weeks after the initial stimulating injections, all of eight rabbits showed some degree of reaction at twenty-four or forty-eight hours to a 1:400 dilution of extract. In the later tests, done in seven of these rabbits, all gave some reaction in twenty-four to forty-eight hours with the extract diluted 1:4,000.

In Vitro Tests With Antisera and Dust Extract E.—All of forty-seven antisera obtained from the injected animals contained precipitins as determined by the interfacial technique. The sera from the five rabbits in the first group produced somewhat less precipitate than the sera from the ten rabbits in the second group and, as would be expected, more precipitate was seen in the later bleedings from all animals.

Forty-five of the forty-seven antisera were tested for their capacity to fix complement with the homologous extract E. Only three antisera failed to fix, but these were so anticomplementary that they could not be used in dilu-

tions lower than 1:80 or 1:100. The remaining forty-two antisera fixed completely with extract diluted 1:4,000 and some with extract diluted 1:64,000 (Table II).

TABLE II. SEROLOGICAL AND SKIN TESTS IN RABBITS WITH DUST EXTRACT E

ANTI-SERUM NO.	DILUTION OF ANTI-SERUM*	TITER† BY COMPLEMENT FIXATION (PER CENT FIXATION)			AGAR DIFFUSION TECHNIQUE (MAXIMAL NO. OF BANDS)		DEGREE OF SKIN REACTIVITY‡ IN IMMUNIZED RABBITS			
							4/14/54		12/13/54	
		100	75	50	BY PLATE	BY TUBE	4,000	400	4,000	400§
6 ₁	30	10,000				2				
6 ₂	40	32,000			4	4	0	++	+	0
	100	16,000								
6 ₃					5					
6 ₄					0					
7 ₁	50	10,000	40,000	100,000						
7 ₂	40	32,000			2	2				
7 ₃					4		++	+++	+	++
7 ₄					1					
8 ₁	100	10,000								
8 ₂	40	32,000		64,000	2	3				
8 ₃					3		+	+++	+	+++
8 ₄					3					
9 ₁	100	32,000	64,000	128,000						
9 ₂	50	16,000		32,000	2	2				
9 ₃					3		+	+++	+	+
9 ₄					3					
10 ₁	100	32,000	64,000	128,000						
10 ₂	40	32,000	64,000		2	3				
	100	32,000		64,000						
11 ₁	25	16,000		32,000						
11 ₂	70	64,000		128,000	3	3	0	+++	±	+
11 ₃					3					
12 ₁	25	16,000								
12 ₂	70	32,000			2	2				
	60	16,000		32,000			+	+++	±	++
12 ₃					2					
13 ₁	100	40,000				2				
13 ₂	40	32,000	64,000		2	3				
	100	16,000	32,000				0	++	+	+++
13 ₃					2					
14 ₁	10	48,000		64,000						
14N¶	10			8,000			0	++		
14 ₂	50	32,000			2	2				
	70	16,000	32,000							
14 ₃					4	2				
15 ₁	10	64,000								
15N¶	10	32,000	64,000							

*Expressed as reciprocal of serum dilution.

†Expressed as reciprocal of antigen dilution.

‡Read at twenty-four hours.

Erythema less than 10 mm., no edema = ±.

Erythema 10 mm. or more, no edema = +.

Erythema 10 mm. or more, slight edema or hemorrhagic area = ++.

Erythema 10 mm. or more, definite edema = +++.

§Expressed as reciprocal of dilutions of dust extract E.

||Test done twice with negative result.

¶Normal serum.

Preimmunization (normal) sera from animals 14 and 15 had some fixing capacity for dust extract. Animal 15, in a dilution of 1:10, fixed almost as well as the immune bleeding in the same dilution. Neither of these produced precipitate in the interfacial test. The preimmunization sera from the remaining eight animals did not fix complement in doses of 0.1 ml. of 1:20 or 1:30 dilution of serum.

With the small amount of C' used, no prozones were observed in any of these tests for fixation, perhaps because the range of antigen dilution was of necessity limited by the anticomplementary nature of extract E. With the use of 12 units of C', permitting a maximal antigen concentration of 1:400, there was a short prozone with three antisera with the weakest of the three dilutions of serum tested. Only one zone of fixation was observed although, as judged by agar diffusion tests to be described below, most of the antisera contained antibody for two or more antigens. It was felt, therefore, that analysis of the antigenic composition of the extract by complement fixation would require very extensive testing and might not be rewarding.

Twenty-six immune sera were tested in tubes³ or plates⁴ with extract E by the agar diffusion technique. All the immune sera gave one or more bands and the third bleeding from animal No. 6 (serum 6₃) gave five, the greatest number observed with any of the sera. The results are shown in Table II. It is interesting to note that sera from the third bleedings from rabbits 11, 12, and 13 produced bands, although these rabbits had not received any injections for thirty-four weeks.

Using serum 6₂ as the reference serum, agar diffusion tests were done in plates to compare the bands produced by eight other antisera from the second group of rabbits. All these gave two heavy bands which joined with the heavy bands produced by 6₂ (reactions of identity⁶). Extract E, therefore, contained two major antigens to which these rabbits responded with production of antibody. The presence of two, rather than a single *major* antigen, as has been described,¹⁰ may be attributable to the difference in the manner in which the extracts were prepared. In addition to No. 6, some animals formed antibody, as judged by band formation in agar, to one or more other antigens present in the extract (see Table I).

Relationship of the Two Major Antigens in Extract E to the Component Responsible for Its Reactivity in the Skin of Dust-Sensitive Persons.—In the following, we proceeded on the assumption that if either of the major antigenic components were responsible for the reactivity of extract E in the skin of dust-sensitive subjects, then the reactive component should go down with the immunologic precipitate. Accordingly, a mixture of 10 ml. of serum No. 14₂ and 10 ml. of extract E 1:40 was made, judged by flocculation tests to represent optimal proportions. After this had stood for three days at 4° to 6° C., it was centrifuged and the supernatant was removed. The tube was wiped out with sterile gauze and the precipitate was washed once with cold sterile saline. Dissociation of this precipitate was attempted by mixing it with 12 ml. of 15 per cent sodium chloride solution.⁷ After this mixture had stood for

eighteen to twenty-four hours at 35° C., it was centrifuged and the supernatant was removed. No precipitate formed when acetone was added to this supernatant to a concentration of 33 per cent. When the amount of acetone was increased to 66 per cent to precipitate any skin-reactive material present, the mixture became slightly opalescent. This precipitate was separated in the centrifuge, saline was added to it, and the insoluble material (rabbit serum protein, etc.) was separated in the centrifuge. The supernatant was inactive in the skin of a suitable dust-sensitive subject. The supernatant of the original mixture described previously, which formed no bands in agar when tested with antiserum, was tested in the skin of the same subject before and after removal of the rabbit serum proteins by heating to 90° C. In confirmation of the findings of Boatner and Efron⁸ and Rimington, Stillwell, and Maunsell,⁹ heating the dust extract under study did not diminish its reactivity in the skin. When diluted to give a final concentration of extract E of 1:40,000, the heated and unheated supernatants reacted to the same degree in the skin as extract E freshly diluted to 1:40,000.

Because of the complex nature of the serologic system, one could not be certain that the mixture of antiserum and antigen had been made in optimal proportions or, indeed, under circumstances wherein a substantial proportion of the antigen in question had been removed. The same type of experiment was carried out, therefore, in the region of clear-cut antibody excess. Mixtures containing equal parts of antiserum and extract E 1:400 were prepared with three different rabbit antisera. After five days at 4° to 6° C., the precipitate present was separated in the centrifuge and the supernatant was saved for testing. In agar diffusion tests the unheated supernatant formed the same number of bands with extract E as the corresponding antisera diluted 1:2, showing that precipitating antibody was in excess in these mixtures. A portion of each supernatant was heated as described previously. Both the heated supernatants, diluted so as to bring the extract to 1:40,000, and extract E, diluted 1:40,000, were equally reactive in the skin of a dust-sensitive subject. The same result was obtained when the experiment was repeated using extract E 1:4,000 instead of 1:400.

These results indicate that the skin-reactive material was not precipitated by the antiserum. That precipitation was complete, or nearly complete, in the first experiment done with equal parts of antiserum and extract E 1:40 was indicated by the failure of the unheated supernatant to form bands in agar diffusion tests when tested with antiserum. In the experiments done with the extract diluted 1:400, the formation of bands indicated a definite excess of precipitating antibody. It was concluded from this experiment, therefore, that the skin-reactive component of extract E was not identical with either of the major precipitating antigens in the extract. This indicates that the *in vitro* standardization of house dust extract by the agar diffusion technique, as suggested by Wodehouse,¹⁰ will be unwarranted unless a correlation between the specifically precipitable antigens and the skin-reactive component of the extract is first established.

To test further the effect of heat, extract E diluted 1:40 was heated for twenty minutes at 60° and 80° C. and each heated sample, together with an unheated control, was tested by agar diffusion in plates. Heating to 60° C. was without effect, but the sample heated to 80° C. formed no bands.

It is evident that the serologic activity of extract E was more easily destroyed by heating than the allergenic activity, since the latter was apparently unchanged after heating for ten minutes at 90° C. Rimington and his associates⁹ reported that the allergenic activity of their purified dust extract was undiminished after thirty minutes of refluxing with 0.5N hydrochloric acid at 100° C.

Tests for complement fixation were done with the heated supernatant from the first mixture made with extract E 1:40 and antiserum 14₂. It was readily shown that heating extract E alone enhanced its anticomplementary activity more than fivefold. (For this reason, fixation tests with the heated extract were not attempted.) However, the heated supernatant from the immune precipitate was, roughly, five times *less* anticomplementary than unheated extract E. This indicates that one, or both, of the major antigenic components of the dust extract was the chief source of its anticomplementary activity. Six antisera, including 14₂, were tested for fixing capacity with this supernatant. There were varying degrees of fixation by the sera, one fixing completely with dilutions of 1:800 to 1:6,400; 14₂ fixed, but not completely, with dilutions of 1:800 and 1:1,600.

It appears, therefore, that removing the immune precipitate and heating the supernatant to 90° C. abolished the capacity of the dust antigen to form bands in agar, lowered the minimal fixing dilution, and markedly decreased anticomplementary activity as compared with extract E heated in the same manner, but did not remove all fixing antigen and left its skin reactivity unimpaired. These findings, coupled with the presence of complement-fixing activity in two normal rabbit sera lacking precipitins, suggest that an antigen was present in extract E which fixed complement and which was not one of the major precipitating antigens. This conclusion cannot be other than tentative in view of quantitative considerations.

Tests With Dust Extract C.—Dust extract C was studied in complement fixation tests, by agar diffusion, and in the skin of dust-sensitive subjects. In agar diffusion, two faint bands were produced in tubes which appeared to be different in character from any of those produced by the homologous extract E; in plates, a single hazy band was produced, which did not join with the two major bands produced by extract E. In complement fixation tests, seven of the nine antisera tested fixed completely with dilutions of extract C ranging from 1:2,000 to 1:32,000. With only two of these seven sera, however, was the minimal fixing dilution of extract C as high as that of extract E, whereas in the other five sera this was two to sixteen times lower. It appears, therefore, that the antigenically reactive substances in extract E had not been concentrated in the course of the preparation and “purification” of extract C. Fur-

thermore, it was distinctly less active than extract E in skin tests. We believe, therefore, that extract C was composed chiefly of relatively inert material.

Tests With Other Extracts.—A concentrated fraction,* which was prepared from freshly ginned cotton in the same manner as that of extract E, fixed complement in low dilution only, produced two very faint bands in agar, and was not reactive in the skin of dust-sensitive subjects. This finding is not surprising in view of the conclusions of others^{11, 12} that aging is required for the development of allergenic activity in cotton and other similar materials.

An extract prepared from dust collected in a large library, and concentrated so that 1 ml. was equivalent to 1 gram of dust, formed bands in agar diffusion tests which were almost as heavy as those formed by extract E, but in skin tests a dilution of 1:30 was approximately equivalent to that of extract E diluted 1:40,000. Here again, allergenicity is not correlated with band formation in agar. A second extract was prepared from dust collected with an electrostatic precipitator in the home of a clinically dust-sensitive asthmatic patient. Two grams was thoroughly extracted by mixing with 30 ml. of water in a Waring Blender. This extract formed two faint bands in agar diffusion tests and it was reactive in the skin of a dust-sensitive subject in a dilution of 1:2.

One of the major serologically active components of extract E and skin-reactive activity were present in simple aqueous extracts of old cotton mattress stuffing prepared by us.

*Tests With Components of the Cotton Plant (*Gossypium barbadense*).*—The possibility that antigens from either the cottonseed or the plant itself might be the source of some of the antigenic components of extract E was also investigated. Extracts of the finely ground seed prepared with Coca's extracting fluid did not react in interfacial tests with five immune sera, but an extract prepared with physiologic saline reacted markedly, not only with these sera but also with three normal rabbit sera. The difference in the behavior of these two extracts perhaps may be attributable to the difference in pH during extraction. Dried fresh leaves and stems of the cotton plant, extracted with physiologic saline, did not react in interfacial tests with four immune sera. These studies are still in progress.

DISCUSSION

The results reported herein indicate that a concentrated aqueous extract of old cotton contains at least five antigenic components, two of which (major antigens) were demonstrable with most of the antisera prepared in rabbits. Strong evidence is presented to indicate that these two major antigens were not responsible for the reactivity of extract E in the skin of dust-sensitive persons. As these antigens and skin reactivity were lacking or present in very small amounts in an extract prepared from freshly ginned cotton, they presumably develop in cotton with the passage of time. They presumably

*Kindly supplied by Dr. Samuel M. Gordon.

must arise from growth of organisms (bacteria, molds), from spontaneous chemical change (oxidation?) in cellulose or other components of the cotton fiber, or as an atmospheric deposit. As allergenic activity appears to be widely distributed in house dust and stuffing from upholstery and old cotton mattresses, decaying vegetation with which the ground out-of-doors is littered likewise might be regarded as a possible source of the same or very similar allergenic activity. Were this the case, however, one might expect that such allergenically active components, being water soluble, would be leached out by rain and lost.

Failure to demonstrate neutralization of the allergen in the experiments in which supernatants from mixtures of antiserum and extract E were tested for allergenic activity cannot be attributed conclusively to lack of antibody in the rabbit sera for the allergenic component. The first of the supernatants obtained from the mixture of equal volumes of serum 14₂ and extract E 1:40 required 500-fold dilution prior to carrying out the skin tests. This might have caused dissociation of a soluble antigen-antibody complex, if such were present, releasing allergenic material. Also, in the later tests, the effect of heat upon such soluble antigen-antibody complexes is not known. However, these possibilities appear to us as an unlikely explanation of the results. On the other hand, the conclusion that allergenic activity was not removed in the antigen-antibody precipitate cannot be questioned. The finding that a dust extract, skin reactive in a dilution of 1:30, formed dense bands comparable to those formed by extract E 1:40, clearly reactive in skin in a dilution of 1:40,000, is further evidence that there is no correlation between the formation of bands with rabbit antisera to extract E and allergenic activity.

It is probable that the two antigens in extract E forming the heavy bands in agar diffusion tests were the ones mainly responsible for fixation in the complement fixation tests. With many of the antisera, these bands formed very close together, which may indicate approximately equal amounts of antibody to each, and this would make it difficult to demonstrate zones of fixation. Those antigens in extract E that formed faint bands near the antigen well were probably present in such low concentrations that they would not have fixed C' in the dilutions of extract E used.

The results of precipitin tests with extracts of cottonseed and cotton plant indicate that they were not a source of antigenic material in extract E.

SUMMARY AND CONCLUSIONS

1. A concentrated aqueous extract of old cotton (extract E) gave rise to precipitating and complement-fixing antibody in rabbits.
2. Extract E contained at least five antigenic components, two major and three minor, and at least one skin-reactive component.
3. These antigenic components were either not present or present in very small amounts in a concentrated aqueous extract of freshly ginned cotton,

which was likewise unreactive in the skin of a dust-sensitive subject; nor were they found in extracts of cottonseed or the leaves and the stems of the cotton plant.

4. Heating extract E to 90° C. enhanced its anticomplementary activity and abolished its capacity to form immunologic precipitates, but did not alter its reactivity in the skin of dust-sensitive subjects.

5. Removal of an immune precipitate from extract E in the presence of excess antibody did not alter the reactivity of the extract in the skin. This and other findings strongly indicate that the skin reactivity of extract E did not reside in the two major antigenic components.

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