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STUDIES ON RAGWEED POLLEN SENSITIZATION IN RABBITS AND GUINEA PIGS¹

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This study is a continuation of an earlier work on sensitization to ragweed-extract and the production of antibodies by means of adjuvants (1). Attempts have been made to gather further information concerning (a) variations of the antibody-level following repeated injections of ragweed extract and adjuvants in rabbits under observation for two years, and (b) the nature of passively sensitizing antibodies produced in guinea-pigs against ragweed-pollen-extract.

(A) MAINTENANCE OF ANTIBODY TITER IN RABBITS

Experimental. Observations of the antibody-titers of the sera of six rabbits which, during an earlier study (1), had received injections of ragweed-pollen-extract, with or without adjuvants² during the course of a year were extended for a second year. Higher concentrations of ragweed-pollen-extract with the same adjuvants (RPEA)³ were employed and the series of injections interrupted by rest periods of 3 to 6 months.

The pollen-extract used in the earlier study contained 2000 protein nitrogen (p.n.) units per ml and single injections consisted of 500-10,000 units. The concentration of pollen added to the adjuvant was raised to 10,000 p.n. units in this study, permitting intramuscular injections of 20,000 to 100,000 per dose. Amounts of adjuvant larger than 0.5 ml were frequently found to cause severe local reaction and, therefore, for higher concentrations of pollen the required dosage of antigen was added to 0.5 ml adjuvant. The rabbits were tested for cutaneous sensitivity at 1 to 3 month intervals, at which time blood was taken and the antibody-titer of the sera determined by the collodion-particle method described by Freund (2) and Cannon and Marshall (3). The details of the methods used in this study were essentially those described in the previous report (1).

Results. In the previous study, the collodion agglutinative titer of the sera of four of the rabbits had reached levels of 1:160 to 1:320 after 8-12 injections of ragweed-pollen-extract with adjuvants (RPEA) 500-2000 units each. In the

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² The antigen-adjuvant mixture was prepared as follows: 8 ml of ragweed-pollen-extract in 10 ml salt solution were added gradually to 10 ml of Aquaphor (trade name of a lanolin-like preparation) in a mortar and blended thoroughly. 20 ml of heavy paraffin oil containing 4 mg killed tubercle bacilli were mixed in the mortar with the combination of pollen-extract and Aquaphor. The procedures were carried out under aseptic conditions and the mixture was kept in sterile rubber-capped vials.

³ Ragweed pollen extract with adjuvant.

present investigation, the same animals received 3 to 5 injections of RPEA (5,000 to 100,000 protein-nitrogen units each) over a period of a year, with 3 to 6 month rest periods intervening. No further rise in titer was observed. Two rabbits, however, whose serum-agglutinative titers were low (1:40 and 1:80) in the preceding study showed increases in titer to 1:320 and 1:160 respectively during this twelve month period.

Injections were frequently followed by a fall and subsequent rise in the agglutinative titer. Such temporary decrease in antibody-concentration following injections of various antigens has been noted by many observers (4). Similar

TABLE 1

Effect of repeated treatment with ragweed-pollen-extract (RPEA) on serum agglutinative titer, cutaneous sensitivity and production of passively sensitizing antibodies in previously immunized rabbits

RABBIT NO.	DATE	AGGLUTINATIVE TITER	SKIN REACTION†					SKIN TEST†	TOTAL DOSAGE RPEA	NUMBER OF INJECTIONS	
			Im-mediate	24 hours							
				1000*	1000*	100*	1*				0.1*
(1944-1945)											
2	12/30/43	1:160	+	++	++	+	+	++	49,000*	5	
	1/18/45	1:120	+	+	±	0	0	0			
14	12/30/43	1:320		++	+	+	+	+	25,000	3	
	1/18/45	1:160	0	++	+	+	0	±			
16	12/30/43	1:320		++	++	++	+	+	205,000	4	
	1/18/45	1:120	0	++	+	±	0	+			
18	12/30/43	1:80		++	++	+	±	0	225,000	5	
	1/18/45	1:160	0	+	+	0	0	+			
20	12/30/43	1:40		+	0	0	0	+	225,000	5	
	1/18/45	1:320	+	+	+	0	0	+			
12	12/30/43	1:160							25,000	3	
	12/15/44	1:160	(Died before 1/18/45)								

* Protein nitrogen units.

† Passive sensitization of the normal guinea pig skin (Chase, 6).

‡ ±: 5-10 mm, red; +: 5-10 mm dark red; ++: 10-20 mm, dark red.

fluctuations were found to occur spontaneously. It appears, therefore, that after injections of ragweed-pollen-extract, as with bacterial proteins (5), once an antibody-concentration is reached which seems to be characteristic for the individual animal, a further rise cannot be consistently induced by repeating the previous treatment.

Maintenance of the agglutinative titer was not accompanied by maintenance of skin-sensitivity. Comparison of the skin-reactions after the first 12 months with those after 24 months showed a decrease in skin-sensitivity in 4 of the 5 rabbits⁴ which were tested.

A successful passive sensitization did not necessarily coincide with a high titer.

⁴ The animals had been skin-tested frequently; the skin tests may have contributed to the maintenance of antibody-titer, and also to the level of skin-sensitivity.

The sera of rabbits 2 and 14 (1/18/45), table 1, did not sensitize the skin of normal guinea-pigs in spite of a high antibody-titer.

B. SENSITIZATION OF THE SKIN OF THE GUINEA PIG AND MAN BY PASSIVELY
SENSITIZING ANTIBODIES

Chase (6) and the authors (1) found that sera of guinea-pigs injected with ragweed-pollen-extract will sensitize the skin of normal guinea-pigs to this antigen. Sherman, Stull and Hampton (7) and Caulfeild (8) reported that the human skin can be sensitized against ragweed-pollen-extract with serum from guinea-pigs injected with alum precipitated ragweed-pollen-extract. Using RPEA as the sensitizing agent, an attempt was made in the present study (a) passively to sensitize normal human as well as guinea-pig's skin with the antibodies produced against this antigen, and (b) to determine a possible relationship between passively sensitizing antibodies and agglutinative titer, skin-sensitivity and anaphylactic shock.

Experimental. Two sets of guinea-pigs were injected with RPEA. The first set (I), 27 animals, received one intraabdominal injection of 0.5 ml (5,000 p.n. units). Of this series, 15 received no additional injections (group B), but the remaining 12 (group A), after a 2½ month interval received 3 more intraabdominal injections of 0.1 ml (1,000 p.n. units) at 5 day intervals. Approximately 2 weeks after the last injection the animals were given skin-tests to determine tissue-sensitivity to ragweed-pollen-extract. Approximately 4 months after the first injection all animals of both groups were bled to obtain serum for the various determinations. They then received a shock dose of 20,000 p.n. units of ragweed-pollen-extract intracardially or intravenously, and their reactions were noted. The surviving animals received a similar injection a month later.

A second set of 20 guinea-pigs (II) received an initial injection of 0.5 ml (5,000 p.n. units) RPEA intramuscularly. Ten of these animals (group B) received no further injections. After a 2 month rest period, the remaining 10 animals (group A) received a series of six further injections of 0.1 ml RPEA intraabdominally at 5 day intervals. Skin-tests were done 3 months after the first injection. Three and one half months following the initial injection all animals were bled and then given a shock dose of RPEA, which was repeated after another month if they had survived.

The sera from the blood samples were preserved with 1:10,000 merthiolate and stored in sterile rubber-capped vials. The sterility of the sera was tested by inoculating tubes of sodium thioglycollate media, which were incubated at 37 C for 48 hours and kept at room temperature in the dark for an additional 72 hours.

The following tests were performed:

(1) Agglutinative titer by the collodion-particle method as in (A).

(2) Skin-test (1)

(3) Passive sensitization of the normal skin of guinea-pigs (Chase 6) and men.

For passive sensitization of the normal guinea-pig's skin, 0.1 ml of the serum, undiluted or diluted, was injected intracutaneously and 24 hours later 1 ml of ragweed-pollen-extract (20,000 p.n. units) was injected subcutaneously into the back at an equidistance from the sites of the serum-injections.

Following preliminary testing for cutaneous sensitivity to ragweed-pollen-extract and to an unrelated antigen (tree pollen or pyrethrum), human recipients giving no reaction to either of these antigens were used in the experiments in passive sensitization. Two recipients were injected intracutaneously with 0.05 ml of 1:10 and 1:100 dilutions of the guinea-pig sera under investigation. The injections were made into the extensor surface of the upper arm, at distances of approximately 5 cm. Six hours later, 0.05 ml (25 p.n. units, as in the preliminary tests) of ragweed-pollen-extract was injected into the sites of the serum-injections. Control tests were done with normal guinea-pig's serum and with an unrelated antigen into one of the sites. The resulting reactions were read after 15 minutes.

Results. The agglutinative titer bore a direct relationship to the number of RPEA injections (table 2). Freund and Bonanto (9b) made a similar observation in rabbits immunized with diphtherial toxoid in water-in-oil emulsion. In group II A, which received a total of 7 RPEA injections over a 3 months period, the lowest titer observed and that in one guinea-pig only, was 1:160; 7 had titers of 1:640 and one a level of 1:1280. In group I A, which had received a total of 4 injections in 3 months, one guinea-pig had an agglutinative titer as high as 1:640, 7 were 1:160 and only one as low as 1:20. The two groups, treated with a single injection, attained much lower antibody levels, only one of those injected intramuscularly (group II B) reaching a titer of 1:160, with 6 at a level of 1:80. The animals which had only one intraabdominal injection (group I A) showed the poorest antibody response, having agglutinative titers, one of 1:80, one of 1:40, two of 1:20 and the rest negative. Thus, when a single dose was given, the intramuscular injection resulted in higher titers than the intraabdominal one.

24 guinea-pigs were used for experiments on passive sensitization of normal guinea-pig skin. The sera of 21 of these animals, injected with ragweed-pollen-extract and adjuvants, transferred sensitizing antibodies to normal guinea-pig skin. No consistent differences could be observed between reactions produced by sera of high and those of low agglutinative titer. The serum of guinea-pig #240 (table 3), which failed to sensitize passively when the agglutinative titer was negative, did produce a positive reaction when the agglutinative titer was 1:20. In three instances, sera of high antibody-titer as measured by the collodion-agglutinative test were also effective in passive sensitization in dilutions of 1:100.

Passive sensitization of the normal human skin was successful in 16 instances with as many guinea-pig sera. Since preliminary observations showed that sera diluted 1:10 caused almost as strong a reaction as undiluted sera, and since a nonspecific serum reaction with undiluted guinea-pig serum sometimes occurred, most of the experiments were done with dilutions of 1:10 and 1:100. Again, no relationship was apparent between intensity of the skin-reaction produced and the agglutinative titer of the injected sera (table 3). In two instances, sera showing no agglutinins induced marked sensitization in a dilution of 1:100 (#1 and #9 in table 3), while others with high agglutinative titers (#281 and #14) contained sensitizing antibodies at no greater dilution than 1:10.

TABLE 2
Serum agglutinative titer and passive sensitization in guinea pigs

SERIES I				SERIES II			
Group A	Skin-reaction*		Agglutinative titer	Group A	Skin reaction*		Agglutinative titer
	Immediate	Delayed			Immediate	Delayed	
245	+	++	20	282	+	++++	160
237	±	±	40	12	±	++++	320
217	+++	++	80	4	+	++++	640
241	+	++	80	10	+	++++	640
138	+++	+++	160	14	++	++	640
243	++	++++	160	250	++	++++	640
287			160	252	±	++++	640
288			160	254	++	++++	640
290			160	281	±	++++	640
298	++	+++	160	16	0	++++	1280
2005	+++	+++	160	Initial injection of 5000 p.n. units of RPEA intramuscularly.			
289			640	2 months later 6 injections of 1000 units RPEA intraabdominally at 5 day intervals.			
Initial injection of 5000 p.n. units of RPEA intraabdominally. 2½ months later 3 injections of 1000 units RPEA intraabdominally at 5 day intervals.							
Group B				Group B			
221	±	+	0	21	0	++	
224			0	9	0	++	0
227			0	253	±	++	±
229			0	1	±	++	80
236	0	++	0	5	0	+++	80
238	0	0	0	7	±	+++	80
240	0	++	0	11	0	++	80
2006	+	+	0	13	0	+++	80
160			0	15	±	+++	80
219	+	±	0	17	0	++	160
162			±	A single injection of 5000 p.n. units of RPEA intramuscularly.			
226			20				
242	0	+	20				
225			40				
299	0	+++	80				
A single injection of 5000 p.n. units of RPEA intraabdominally.							

* ±: 5-10 mm, red; +: 5-10 mm, dark red; ++: 10-20 mm, dark red; +++: necrotic, less than 10 mm; ++++: necrotic, more than 10 mm.

The relation of passively sensitizing antibodies to skin-sensitization was studied in regard to 1) immediate, and 2) delayed skin-reaction.

1) Immediate skin-reaction developed in most of those animals which had received several injections of RPEA (group B of table 2) and seems to indicate a high degree of skin-sensitivity. The sera of all these animals contained

TABLE 3

Serum-agglutinative and passive sensitization tests in normal guinea pigs and in man

G. P. NO.	COLLOIDION AGGLU- TINATIVE TITER	PASSIVE SENSITIZATION TO					
		Guinea pig serum dilutions			Human serum dilutions		
		Undiluted or 1:2	1:10	1:100	1:2	1:10	1:100
2006	0	++		0	+	+	
236	0	++		0		+	
9	0					++++	+++
1	0					++++	+++
221	0	++					
222	0	+					
219	0	0	0				
229	0	++					
240	0	0	0	0			
240	20	+++	+				
253	10					++	+
226	20	++					
242	20	++	++	0			
*245	20	+++		0	++	++	+
237	40	+++			++	++	+
252	40		0	0			
5	40				+	+	+
9	40		+++	++			
†4	40		+++	+++			
225	40	+++	+				
299	80	+++		0	+++	+	
7	80					±	0
15	80					±	0
217	80	+++					
241	80	+++	0			+	+
138	160	+++	++	++	+	+	+
298	160	++		++		+++	
243	160	++	++				
288	160	++	0	++			
2005	160	++					
241	320		++				
*245	320		++				
289	640	+++					
†4	640		0	0		+++	+++
281	640					++	0
14	640					++	0

* Tested with titers 1:20 and 1:320.

† Tested with titers 1:40 and 1:640.

±: 5-10 mm; +: 10-15 mm; ++: 15-20 mm; +++: 20-40 mm; ++++: 40-50 mm.

passively sensitizing antibodies. However, other animals developed sensitizing antibodies but showed no immediate skin-reaction.

2) Some of the animals which had received 3, and all of those which had received 6 additional intraabdominal injections developed hemorrhagic and necrotizing skin-lesions at the site of skin reaction which sloughed off and did not

heal for many weeks. The concentration of passively sensitizing antibody did not correspond to the severity of skin-reaction, as shown by the fact that the serum of some of these animals produced successful passive sensitization at a dilution of 1:10 but not at a dilution of 1:100. Otherwise, no difference could be observed between reactions produced by sera of high and those with low agglutinative titer.

In series I, group A, 4 of 12; in group B, 7 of 15; in series II, group A, 1 of 10 and in group B, none of 10 guinea pigs died of anaphylactic shock. The reactions of other guinea-pigs varied from none to severe. Thus, it appears that there is no correlation between passively sensitizing antibody concentration and severity of anaphylactic shock. Nor was a relationship apparent between agglutinative titer and susceptibility of animals to anaphylactic shock.

DISCUSSION

An Aquaphor-paraffin oil adjuvant with or without killed tubercle bacilli has been shown by Freund and co-workers (9) to be effective for sustaining serum antibody levels and skin-sensitization for six months or more, when combined with horse serum, diphtheria toxin, typhoid bacilli and other antigens. In the present investigation, it was found to be equally effective when combined with ragweed-pollen-extract. No significant fall in collodion agglutinative titer was observed when injections were discontinued for periods of 3 to 6 months.

These findings suggest the potential value of similar studies on human patients with hay fever. The adjuvant used in this work contained killed tubercle bacilli and may cause cold abscesses. Henle and Henle (10) however, without the addition of tubercle bacilli, have successfully used a water-in-oil emulsion combined with influenzal vaccines in vaccinating human beings against influenza.

If a high titer of induced antibodies is essential for clinical improvement and if the antibody-titer could be kept as high and as constant in human subjects as it was in rabbits by semi-annual injections, the adjuvant method might lead to a great simplification of hay-fever therapy. It is possible, however, that the application of the adjuvant method might lead to increased clinical symptoms of allergy. Antipollen antibody-titers can be determined in the sera of humans by the collodion-agglutinative method used in this investigation. This has been shown by Cohen et al (11), whose suggestive results were confirmed by the present authors in unpublished observations on patients of the New York Hospital Allergy Clinic.

In most of the animals antibodies capable of sensitizing human skin were found. These antibodies usually appeared earlier than collodion-agglutinative antibodies. The sensitizing antibodies did not parallel agglutinative titers, and in some instances showed the highest concentration when no agglutinins were present. When sera from human subjects with hay fever were filtered through a Seitz filter, agglutinative antibodies were retained whereas passively sensitizing antibodies passed through the filter.

The atopic, sensitizing reagins that occur in man suffering from allergic diseases are thermolabile and have a high affinity to the human skin. In these respects

reagins differ from the usual induced antibodies (anaphylactic antibodies, precipitins) of lower animals and man (12). It would have been of interest to determine the heat-lability and also the affinity to human skin of the antibodies found in the sera of guinea-pigs and rabbits capable of sensitizing the human skin to ragweed-pollen-extract. It is interesting to note that both reagins and neutralizing antibodies against ragweed were found in cattle by Weil and Reddin (13).

SUMMARY AND CONCLUSIONS

1. Rabbits were injected with ragweed-pollen-extract in water-in-oil emulsion containing killed tubercle bacilli, and observed over a period of two years. Repeated injections were found to produce more vigorous sensitization and antibody-formation than a single injection. Once a serum-antibody-level is reached which is characteristic for the animal it is usually maintained for six months without injection.

2. Guinea-pigs injected with ragweed-pollen-extract with adjuvants were shown to develop antibodies capable of passively sensitizing normal human skin. These antibodies seem to be quantitatively unrelated to the collodion-agglutinative titer. No relation was observed between them and the degree of skin-sensitivity, nor to susceptibility to shock.

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