

The effect of alum-precipitated pyridine ragweed extract on guinea pigs

II. Comparison of passive cutaneous anaphylaxis with Arthus reactivity after single low dose

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To investigate further the enhanced antigenicity of alum-precipitated pyridine (APPR) as compared to aqueous ragweed extract (ARE), passive cutaneous anaphylaxis (PCA) experiments were performed using sera obtained from the guinea pigs prior to the studies for Arthus reactivity previously reported.¹ The APPR immunized animals produced a greater total number of Arthus reactions, whether moderate or maximum, and the sera from these same animals gave a larger total percentage of positive PCA tests, 39 of 120 (32.5) per cent, than did the ARE immunized animals, 5 of 36 (13.8 per cent). Chi square for this difference was 4.737 and is significant at the 5 per cent level. The APPR immunized animals appeared to develop two types of antibodies more consistently than the ARE immunized. Their sera gave positive PCA reactions earlier after immunization and with lower single doses of APPR than did ARE immunized animals. With immunizing doses of 10 μ g of protein N or below, antibodies persisted longer in APPR immunized animals. On day 136, 10 of 10 animals immunized with this dose of APPR gave positive Arthus reactions and their sera produced 7 of 10 positive PCA tests. On the other hand, none of the ARE immunized animals developed Arthus reactions nor did their sera produce positive PCA tests.

In a preceding paper¹ the Arthus reaction was used as an index of the comparative effect of single minimal immunizing doses of 0.01 to 100.0 μ g protein nitrogen of alum-precipitated pyridine ragweed extract (APPR) and aqueous ragweed extract (ARE) in similar groups of guinea pigs. Intradermal challenge, done at regular intervals from 5 to 136 days, consisted of 0.1 ml. containing 0.1, 1.0, and 10.0 μ g protein N of ARE and diluent controls. An analysis of the

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This investigation was supported in part by Public Health Service Training Grant T1 HD 62-0481 from the National Institute of Child Health and Human Development, Public Health Service, and in part by a grant from Dome Chemicals, Inc., New York, N. Y.

Presented in part at the Annual Meeting of the American Academy of Allergy, San Francisco, Calif., Feb. 11, 1964.

Received for publication June 11, 1965.

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results showed that the Arthus reactions obtained with the APPR immunized animals were larger and occurred more frequently than those in animals immunized with ARE. Furthermore, it was pointed out that a single immunizing dose of APPR as low as 0.1 μ g protein N could induce this reaction and a response could be demonstrated even 136 days after immunization. Just prior to the skin testing of these animals, blood was collected by cardiac puncture and the separated sera were stored at -20° C.

This report presents the results of passive cutaneous anaphylaxis (PCA) experiments done with these sera, to investigate further the degree of antigenicity of APPR as compared to ARE. Because many animals from both antigen groups developed PCA reactions in addition to these Arthus reactions, it is evident that at least two immunological components were induced.

Others^{2, 3, 4} have indicated that the Arthus reaction and the passive cutaneous anaphylaxis reaction are produced by different antibodies. This report suggests that the APPR immunized animals are capable of developing these two types of antibody responses more consistently. The ARE immunized animals showed markedly less tendency both to develop a good Arthus reaction and to give a positive PCA test with serum from the same animal.

MATERIALS

Introduction

The animals used, the method of immunization, and the procedure for collecting and preserving the antisera obtained prior to skin testing have been described.¹

Antigens*

The stock solution of alum-precipitated pyridine ragweed complex (APPR) (Lot 2-S), as reported by Fuchs and Strauss,⁵ and the 50 per cent glycerinated aqueous extract of defatted ragweed pollen (ARE) have been described.¹

A phenolated aqueous ragweed extract (AREP) (Lot QF 11-2), containing 50 per cent high and 50 per cent low ragweed, 0.4 per cent phenol, and 630 μ g protein N per milliliter was used for all dilutions of antigen in passive cutaneous anaphylaxis. Dilutions were made with 0.85 per cent saline.

Control

Normal guinea pig serum, diluted 1:2 with 0.85 per cent saline solution, served as the control intradermal test material used on each animal for PCA tests.

METHODS

Passive cutaneous anaphylaxis

All available sera collected from guinea pigs immunized with ARE or APPR were analyzed for antibody content by the method described by Ovary.⁶ Twenty-four hours before testing, guinea pigs weighing 250 grams were shaved with an

*All ragweed extracts were supplied and standardized by semi-micro-Kjeldahl methods by Margaret Strauss, Director, Allergen Laboratories, Dome Chemicals, Inc., New York, N. Y.

Oster clipper No. 40 blade. Ten intradermal injections of 0.1 ml. of a 1:2 dilution of all test antisera were made in the flanks of the guinea pigs. The sera obtained from the animals that had given maximum Arthus reactions¹ were diluted twofold until a negative end point was reached. Each serum dilution was tested on 3 or 4 guinea pigs. Each animal had a control intradermal injection of normal guinea pig serum diluted 1:2. After a latent period of 5 hours the animals were challenged intracardially with 1.25 ml. of a mixture containing 520.0 μ g protein N of AREP in 1 ml. of 0.85 per cent saline and 0.25 ml. of 1 per cent Evans blue dye. The reactions were read in 15 to 30 minutes after challenge and the cross diameters of the "bluing reactions" were recorded and photographed. In all tests failing to show gross bluing in excess of 5 mm., the skin was reflected on the sacrificed animal. If the bluing was present on the undersurface of the skin and was in excess of 5 mm., it was considered positive and also was photographed. Inasmuch as concentrated sera (even before immunization) have been known to cause bluing reactions, the reactions were graded as zero if the difference in the diameters between the control (normal guinea pig serum) and the antisera, both diluted 1:2, was less than 5 mm.^{6, 7}

Code for classifying intradermal reactions

Four-hour skin reactions resulting in an average of cross diameters of less than 10 mm. erythema and 8 mm. of induration were considered negative.

Moderate reactions ranged from $\frac{10 \text{ to } 16}{8 \text{ to } 14}$ erythema/induration, in millimeters, with a change in skin thickness ranging from 7 to 17×10^{-1} mm. Maximum reactions ranged from $\frac{17 \text{ to } 35}{15 \text{ to } 32}$ erythema/induration, in millimeters, with the change in skin thickness ranging from 18 to 28×10^{-1} mm.

EXPERIMENTAL RESULTS

I. Passive cutaneous anaphylaxis

A. Photograph of representative PCA test. The results of a representative passive cutaneous anaphylaxis experiment are presented in Fig. 1. In this experiment PCA was done to demonstrate the antibody content of an antiserum collected on day 50 from a guinea pig immunized with 30.0 μ g protein N of APPR. The control test is on the left and then in sequence from left to right are the blue reactions produced by 1:2 and 1:4 dilutions of antisera. The 1:8 through 1:256 were also positive for this same antiserum.

B. PCA tests done on sera collected prior to Arthus reactions from guinea pigs immunized with APPR and ARE.

1. INTRODUCTION. In Table I are presented all the PCA results obtained on sera collected from 266 guinea pigs prior to the production of Arthus reactions in these same animals. The test sera were separated into groups depending upon the Arthus reaction size. PCA results were likewise compared with Arthus reaction size. The results of these Arthus reactions, although previously reported,¹ are presented in this table in order to compare the types of reaction produced.

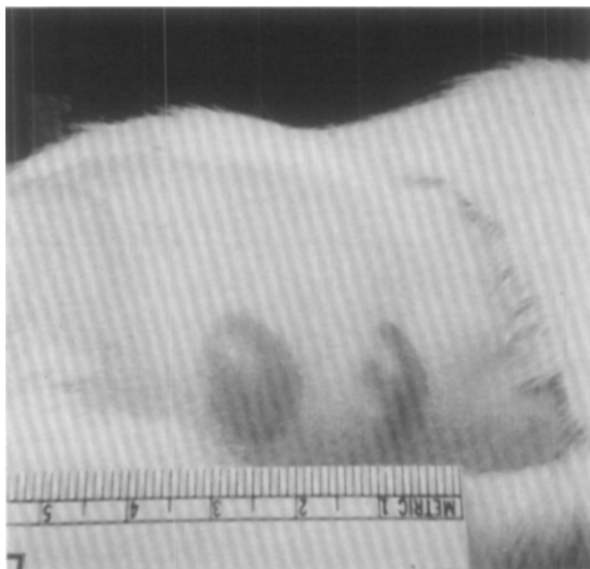


Fig. 1

PCA reactions that followed AREP intracardially, with the use, intradermally, of day 50 serum from a guinea pig immunized with 30.0 μg protein N of APPR. Left, normal serum 1:2; middle, antiserum 1:2; right, antiserum 1:4 dilution.

2. DAY 7. APPR sera gave one positive PCA test from an animal with an immunizing dose as low as 0.1 μg of protein N, but 2 of 5 sera gave a positive PCA when the immunizing dose was at the upper limit, 100.0 μg . With the 100.0 μg immunizing dose of APPR there was one animal that gave a negative Arthus but a positive PCA. When ARE was the immunizing antigen, all sera produced by these animals gave negative PCA tests regardless of immunizing dose.

3. DAY 15. Worthy of note on this day was the fact that at the 0.1 μg dose level 2 of 12 of the APPR animals gave a positive PCA. One of the two sera giving a positive PCA came from an animal that had given a moderate Arthus reaction and one from an animal that had given a negative Arthus reaction. On the other hand, with the ARE immunized animals at this low dose, there were none that gave a positive Arthus and none whose sera produced a positive PCA. When the immunizing dose of APPR was 10.0 μg of protein N, 4 of 13 sera produced a positive PCA test and 3 of these 4 came from animals that had given a maximum Arthus reaction and the other serum came from an animal that had given a moderate Arthus reaction. At this same dosage level of antigen, 6 of 6 ARE animals produced sera that gave a positive PCA, and 5 of these animals had a negative Arthus reaction while one had a moderate Arthus reaction. When the dose of antigen was 30.0 μg of protein N, 3 of 5 of the APPR immunized animals produced sera that gave a positive PCA and all of these 3 animals also had developed positive Arthus reactions, two of which were maximal in size. However, when the immunizing dose of ARE was 30.0 μg of protein N,

Table I. Comparison of Arthus reaction size with PCA results produced with sera from same animals immunized with a single low dose of either APPR or ARE

	Immunizing dose (μ g protein N)	Immunizing antigens						
		Alum-precipitated pyridine ragweed				Aqueous ragweed extract		
		Arthus reaction to 10 μ g protein N ARE	Passive cutaneous anaphylaxis		Arthus reaction to 10 μ g protein N ARE	Passive cutaneous anaphylaxis		
			Positive	Negative		Positive	Negative	
Day 7								
	0.1	Mod. 2*	2		—†	—	—	
		Neg. 3	1	2				
	1.0	Max. 3		3	Neg. 4		4	
		Mod. 3		3				
	10.0	Max. 4		4	Neg. 5		5	
	30.0	Max. 2		(2)‡	Neg. 6		4	
		Mod. 11	1	(10)			2 qns	
	50.0	Max. 3		3	Neg. 5		5	
		Mod. 1		1				
	100.0	Max. 3	1½	2				
		Mod. 2		2	Neg. 4		4	
		Neg. 1	1¹					
Day 15								
	0.1	Mod. 5	1¹	4	Neg. 6		6	
		Neg. 7	1¹	6				
	1.0	Max. 2	2					
		Mod. 2		2				
		Neg. 2	1²	1	Neg. 5	1	4	
	10.0	Max. 9	3¹	6	Mod. 1	1²		
		Mod. 4	1	3	Neg. 5	5		
	30.0	Max. 4	2²	2	Max. 2		2	
		Mod. 1	1² 1:64		Neg. 3		3	
	100.0	—	—	—	Max. 1		1	
					Mod. 1		1	
					Neg. 4	1¹	3	
Day 28								
	1.0	Max. 1		1	Mod. 1		1	
		Mod. 3		3	Neg. 4	2	2	
		Neg. 1		1				
	10.0	—	—	—	Max. 2		2	
					Mod. 1		1	
					Neg. 2		2	
Day 34								
	30.0	Max. 7		7	Max. 2		2	
		Mod. 1	1³ 1:8		Mod. 3		3	
					Neg. 2	1² 1:16	1	
Day 50								
	1.0	Max. 3		3	Neg. 6	1	5	
		Mod. 1		1				
		Neg. 2	1	1				
	10.0	—	—	—	Max. 2		2	
					Mod. 2		2	
					Neg. 2	1	1	
	30.0	Max. 5	{ 1	3	Max. 1		1	
			1² 1:32					
		Neg. 1	1² 1:256		Mod. 1		1	
					Neg. 3	3²		

*Figures are number of animals. All reactions in each group are listed. If no negatives, there were none.

†Means no animals were Arthus tested in this category. Only the sera from animals on which comparable Arthus reactions were performed are included in this table.

‡Parentheses around figures mean the sera for that group were pooled when tested.

§PCA tests were positive in 3 of 3 animals unless marked by an exponent; 1 means one of three, 2 means two of three, and 3 means one of four PCA test animals gave positive results.

||End points of positive PCA tests were with sera dilutions 1:2 unless otherwise specified.

Table I. *Cont'd*

	Immuniz- ing dose (μ g pro- tein N)	Immunizing antigens					
		Alum-precipitated pyridine ragweed			Aqueous ragweed extract		
		Arthus reaction to 10 μ g pro- tein N ARE	Passive cutaneous anaphylaxis		Arthus reaction to 10 μ g pro- tein N ARE	Passive cutaneous anaphylaxis	
			Positive	Negative		Positive	Negative
Day 82							
	1.0	Mod. 4	1 ¹	3	—	—	—
		Neg. 5	1 ¹	4	—	—	—
	10.0	—	—	—	Max. 4	—	4
					Mod. 2		2
					Neg. 2	1 ¹	1
	30.0	Max. 8	(7) ¹ 1:4	1	Mod. 1		1
					Neg. 5		5
	100.0	—	—	—	Max. 4	3	1
					Mod. 1		1
					Neg. 3	1	2
Day 136							
	1.0	Max. 4		4	—	—	—
		Mod. 1		1			
	10.0	Max. 8	6 ²	2	Neg. 6		6
		Mod. 2	1 ¹	1			
	30.0	Max. 8	5 ¹	3	Mod. 2		2
		Mod. 1	1 ² 1:32		Neg. 4	2 ¹	2
	100.0	Max. 3	3 ¹		Mod. 3	1 ¹ 1:16	2
					Neg. 2	{ 1 ² 1 ² 1:4	

5 of 5 of the sera gave a negative PCA and yet 2 of the 5 animals from which the sera came gave maximal Arthus reactions.

A similar comparison of the Arthus reactions developed on days 28 and 34, and the PCA reactions to the sera that these same animals produced are presented in Table I.

Perhaps the most significant findings were in sera from day 50 when the immunizing dose of both antigens was 30.0 μ g of protein N. Three of 6 of the APPR animals produced sera that gave positive PCA tests and in one of these 3 the end point was at 1:256 dilution of the serum. An interesting fact, too, is that the animal that produced this serum gave a negative Arthus reaction and the 2 other sera came from animals that gave a maximum Arthus reaction. Three of 5 sera collected on day 50 from animals immunized with 30.0 μ g protein N of ARE gave a positive PCA and all 3 of the animals from which these sera were derived gave negative Arthus reactions.

The findings for day 82 are presented in Table I. Seven of 8 sera from APPR animals immunized with 30.0 μ g of protein N that developed maximal Arthus reactions gave positive PCA tests. On the other hand, 6 of 6 sera from ARE animals immunized with the same dose gave negative PCA tests. Five of these 6 animals had had negative Arthus reactions and one was a moderate reaction. However, when the immunizing dose of ARE was raised to 100.0 μ g of protein N, 4 of 8 sera produced a positive PCA test and one of these came from an animal that had developed one of the only 3 maximal Arthus reactions in the ARE immunized group under discussion.

The differences between the PCA results for the APPR immunized animals and ARE immunized animals were the least on day 136. The main difference was that when the immunizing dose of ARE was as low as 10.0 μg of protein N, all of the sera produced negative PCA tests and none of the animals from which these sera came developed positive Arthus reactions. At the 100.0 μg protein N dosage level the differences between the PCA results obtained with APPR and ARE were the least (Table I).

DISCUSSION

The technique of intravenous injection of a dye, trypan blue, to reveal the increased permeability of the minute vessels in the skin of the guinea pig during cutaneous anaphylaxis was first used by Ramsdell.⁸ Similar reactions were observed by Chase⁹ in the skin of the guinea pig by simply injecting antibody and 24 hours later injecting antigen in the same skin test site. It was the opinion of both Chase and Ovary¹⁰ that this change in the skin was produced by the release of histamine as a result of a localized cutaneous antigen-antibody interaction. Subsequently quantitative studies were performed with transferred antibody which Ovary¹⁰ in 1953 called passive cutaneous anaphylaxis (PCA).

The PCA reaction requires a latent period between the injection of antibody and injection of antigen. Ovary and Bier¹¹ attributed the latent period to the time necessary for the anchorage of antibody to the cells in the guinea pig skin. These authors also showed that the latent period was inversely proportional to the amount of antibody present. The average latent period was 4 to 6 hours. Because the immunization doses in the experiments presented here were minimal,¹ it was assumed that the antibody content of the sera might be low, so the latent period chosen for all experiments in this report was 5 hours.

Since the great variability in the reactivity of different guinea pigs is well known, an average of 3 and sometimes 4 guinea pigs were used in the PCA test for each serum.

Ovary^{2, 12} states that, if the aim is detection of antibody, it is necessary to use a large excess of antigen, and he showed that the amount of antigen necessary was inversely proportional to the amount of antibody present. He¹⁰ further stated that, since a great excess of antigen is not inhibitory, it is safe to use 10 to 100 times more than necessary for maximal PCA reaction and that threshold amounts of antibody react faster with excess of antigen.¹³ Because of these facts accumulated by previous investigators of the effect of the amount of antigen in relation to maximal production of PCA, it was decided that in all these experiments it would be wise to use 520.0 μg protein N of AREP. Since substances extracted from whole ragweed pollen have a relatively low molecular weight¹⁴ and the average amount of antigen used in PCA by others for challenge is 200.0 to 300.0 μg protein N, it was believed that the amount used in these experiments would provide a great excess and therefore a maximal PCA for the amount of antibody that might be present.

Ovary¹² has shown that PCA is a very useful method of detecting antibody because of the high degree of sensitivity of the test and the very small amount of antibody needed. Ovary^{6, 11} has shown that this test in guinea pigs can detect 0.003-0.01 μg antibody N in 0.1 ml. of serum.

These experiments have indicated that there are noticeable differences between the immunological responses of the ARE and APPR immunized animals to the Arthus reaction¹ and passive cutaneous anaphylaxis produced by the sera of these same animals at the various time intervals after single low dose immunization. The findings produced by the PCA tests on all available sera diluted with saline 1:2 or above from both APPR and ARE immunized animals suggest that APPR induces, more consistently than ARE, two types of antibodies. One of these produces a much greater number of Arthus reactions (in this study 120 in 142 animals as compared to 31 in 124 ARE immunized guinea pigs) and the other antibody is capable of inducing positive PCA reactions. Forty-seven of 142 PCA tests were positive when the sera used came from guinea pigs immunized with APPR and 26 of 124 PCA tests performed were positive when the sera used were from the guinea pigs immunized with ARE. The APPR immunized animals had a significantly greater tendency to produce both a maximum Arthus reaction and a positive PCA as compared to the ARE animals. Thirty-one of 142 or 21.9 per cent of the APPR immunized animals developed both a maximum Arthus reaction and a positive PCA test, whereas only three of 124 or 2.4 per cent ARE immunized animals had a maximum Arthus and a positive PCA (Table I).

The sera of 24 of 88 (27.1 per cent) of the ARE immunized animals with negative Arthus reactions produced sera that gave a positive PCA, and 7 of 22 (31.8 per cent) of the sera from the APPR immunized animals with a negative Arthus reaction produced a positive PCA. Chi square for this is 0.1796, so there is no significant difference at the 5 per cent level in the ability of the negative Arthus reactors to produce a positive PCA whether immunized with ARE or APPR. Eighty-eight of the 124 ARE immunized animals (70.9 per cent) studied had a negative Arthus, whereas 22 of the 142 APPR immunized animals (15.4 per cent) had a negative Arthus reaction. Seventy-seven of the 120 APPR immunized animals that gave a positive Arthus reaction gave one in the maximum range, and only 18 of 36 of the ARE immunized animals produced this.

Benacerraf, Ovary, Bloch, and Franklin,³ immunizing guinea pigs with purified antigens, have produced two distinct antibodies, both with sedimentation coefficients of 7S but different electrophoretic mobilities, that is, guinea pig 7S gamma-1 and 7S gamma-2 globulin. Ovary, Benacerraf, and Bloch¹⁵ state that guinea pig 7S gamma-1 antibody has the capacity to transfer PCA activity in guinea pigs while 7S gamma-2 antibody will specifically block the PCA test in proper concentrations. Their immunological systems differed in that they used highly purified antigens, while the aqueous ragweed extract is known to contain 10 or more antigens.¹⁶ Although we did not determine electrophoretic mobility on the sera involved in this study, it probably can be assumed that the APPR produced in the guinea pigs, in most instances, antibody to each of these two fractions. Bloch, Kourilsky, Ovary, and Benacerraf⁴ found 7S gamma-2 antibody effective in conferring passive Arthus reactivity in the guinea pigs. Presumably there was more of this antibody produced in the guinea pigs by APPR immunization than by ARE since we had many more maximum Arthus reactions in the APPR immunized group of animals than in the ARE immunized. However, it would not appear that whatever had developed had been

sufficient to block the PCA reaction which evidence suggests is produced by a different antibody.⁴ It is germane to point out though that Ovary, Benacerraf, and Bloch¹⁵ in their inhibition experiments used specific concentrations in their mixtures of 7S gamma-1 and 7S gamma-2 whereas in these experiments there was undoubtedly a biological variation in the amounts of each of these two fractions rather than a fixed amount of each type of antibody at the skin test site. The multiplicity of antigens in the ragweed immunological system also undoubtedly was a factor.

On day 7, the APPR immunized animals were able to produce sera with sufficient antibody to give a positive PCA in 1:2 dilution in 3 of 5 tested with as low an immunizing dose as 0.1 μ g of protein N. Two of these 5 animals also gave a positive Arthus reaction.

It is of particular interest to note that the sera from animals immunized with 30.0 μ g protein N of APPR produced PCA end points in 1:64 dilution on day 15, 1:256 dilution on day 50, and 1:32 dilution on day 136. On the other hand, all the sera from animals that gave a maximum Arthus reaction and were immunized with ARE gave negative PCA except on day 82, and they were from animals that received the highest immunizing dose. On day 136, 3 of 5 sera from animals immunized with ARE gave a positive PCA; one in 1:4 dilution (from a guinea pig that gave a negative Arthus reaction) and one in 1:16 dilution, suggesting these animals had in their sera more of the fraction responsible for PCA activity and less of that fraction responsible for Arthus reactivity. There were only 3 in the total ARE immunized group of animals that developed a maximum Arthus reaction and only 5 of 124 ARE immunized animals that developed an Arthus reaction of any size.

At an immunizing dose of ARE as low as 10.0 μ g of protein N, antibody apparently did not persist for as long a duration as it did when APPR was the immunizing antigen. On day 136, 10 of 10 APPR immunized animals had moderate or maximum Arthus reactions and 7 of these 10 animals produced sera that gave a positive PCA, whereas with the same immunizing dose, or 10 μ g of protein N of ARE, 6 of 6 animals gave negative Arthus reactions and all the 6 animals produced sera that gave negative PCA tests.

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