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THE IMMUNOCHEMISTRY OF ALLERGENS
VIII. Precipitin Formation and Passive Transfer Reactions with Allergenic Proteins from Cottonseed and Castor Beans[1, 2]

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The proteic-polysaccharidic fraction, CS-1A, from cottonseed has been shown to contain a non-dialyzable polysaccharide and an allergenic protein. This protein diffuses readily through Cellophane membranes that are essentially impermeable to crystalline ovalbumin (1). The diffusibility of the allergenic protein of CS-1A denotes a significantly smaller molecular size than has been commonly held essential to antigenicity. Nevertheless, the anaphylactogenic capacity of the allergenic protein of CS-1A was readily demonstrable in guinea-pigs (2). A similar proteic-polysaccharidic substance, CB-1A, was later isolated from castor beans (3). This fraction resembled the corresponding fraction CS-1A from cottonseed in physical and chemical properties and also, with respect to allergenic and anaphylactogenic activity. Hence, both CS-1A and CB-1A are potent anaphylactogens but judged by their diffusibility they resemble haptens in molecular size rather than typical protein-antigens. Accordingly, additional evidence was sought to distinguish the antigenic character of these fractions.

This report deals with a study of precipitin-formation in rabbits induced by administration of CS-1A, CB-1A, and a typical protein-antigen, crystallized ovalbumin. The capacities of the resulting antisera to induce passive sensitization in guinea-pigs were also compared. The first series of experiments was conducted in September 1938 and included only CS-1A and crystalline ovalbumin. The second series was performed in September 1943 and included CS-1A, CB-1A, and ovalbumin.

EXPERIMENTAL

First series

Three rabbits (New Zealand White) weighing about 2 kg were treated in the following manner:

Rabbit no. 1 was given a series of injections of 4 per cent ovalbumin-solution. The schedule of injections was essentially the same as that used by Walzer and Grove (4).

Rabbit no. 2 was treated according to the same schedule with a 4 per cent solution of CS-1.[4]

[1] Contribution from Allergen Investigations, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

[2] For Paper VII of this series see Coulson, Spies, and Stevens, J. Allergy 1945, **16**, 176–183.

[3] Now with Western Regional Research Laboratory, Albany, California.

[4] CS-1 and CS-1A are identical fractions of cottonseed obtained by slightly different methods (5).

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Rabbit no. 3 received subcutaneous injections in 5 separate sites on the same day. One ml of an alum-precipitated, 4 per cent solution of CS-1 was injected into each site.

On the seventh day after the final subcutaneous injection, rabbits no. 1 and no. 2 were bled. Sera were preserved by the addition of phenol to a final concentration of 0.25 per cent.

Rabbit no. 3 was sacrificed for serum on the twenty-second day after injection of the alum-precipitated solution of CS-1.

Precipitin-tests were conducted by the antigen-dilution method. One-tenth ml of serum was dispensed into each of a series of 3 x 40 mm tubes. An equal volume of tenfold serially diluted antigen was stratified over the serum. Readings were made after 30 minutes, and again after 2 hours in the incubator. The contents of each tube were then mixed and the tubes were stored in the refrigerator. Final readings were made after 24 hours.

The optimal concentration for the ovalbumin antigen, when tested with the antisera for ovalbumin, was about 1 part ovalbumin in 2500 parts of solution. An appreciable precipitate formed at antigen-dilution of 1:25,000 and a trace at 1:250,000. The diluted ovalbumin gave no precipitate with normal rabbit-serum.

Precipitins were not demonstrable in either of the two antisera from the 2 rabbits previously injected with CS-1.

The capacity of each of the 3 antisera to sensitize by passive transfer was estimated by determining the minimal quantity of antiserum that induced fatal anaphylactic sensitization in guinea-pigs. For this determination guinea-pigs weighing approximately 300 g were arranged in 3 uniform groups of 5 animals. Each animal of a group was given a single, 1-ml dose of diluted antiserum by intra-abdominal injection. Forty-eight hours later 1 mg of the homologous antigen in 0.5 ml of saline was injected intravenously. The data recorded in table 1 are typical of the results obtained by the passive-transfer technic.

The minimal passive sensitizing dose (MSD) of the ovalbumin-antiserum was equivalent to 0.04 ml of undiluted serum. The MSD of the antiserum for CS-1 (rabbit no. 2) was 0.35 ml. An injection of 2.0 ml of undiluted antiserum from rabbit no. 3 (alum-precipitated CS-1) was required to establish fatal sensitization.

Second series

For preparation of antisera in the second series of experiments, 2 rabbits (nos. 12 and 13) were treated with CS-1A; 2 rabbits (nos. 14 and 15) were treated with ovalbumin; and 2 rabbits (nos. 16 and 17) were treated with CB-1A. The respective antigens, in 4 per cent solutions, were administered as described in the first series.

To determine the effect of the length of the incubational period on precipitin-formation, blood for preparation of antisera was collected on the second, eighth, and forty-seventh day after the last injection of antigen. One animal from each of the 3 groups was bled from the marginal ear-vein on the second day after the final injection. The resulting antisera were designated 12A, 15A, and 17A. All of the animals were bled on the eighth day following the final injection, for

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preparation of antisera no. 12B, 13B, 14B, 15B, 16B, 17B. Finally, the 2 ovalbumin-treated rabbits were bled again, 47 days after the final injection, for preparation of antisera nos. 14D and 15D.

To determine the effect of additional antigenic stimulation on precipitin-formation the 2 rabbits first treated with CS-1A and the 2 rabbits first treated with CB-1A were given a second course of injections. This second course was begun 4 weeks after the final injection of the first course. The first dose of the second course was an intra-abdominal injection of 0.25 ml of the 4 per cent solution of the antigen. Two days later a 1-ml dose of antigen was injected subcutaneously.

TABLE 1

Determination of MSD of rabbit-antisera by passive transfer to guinea-pigs*

ANIMAL NO.	SENSITIZING DOSE	RESULT OF CHALLENGING INJECTION
	Rabbit vs ovalbumin. Serum 1	
	ml†	
550	0.02	Severe
554	0.04	Fatal, 3 min
548	0.06	Fatal, 3½ min
544	0.08	Fatal, 3 min
545	0.10	Fatal, 4 min
	Rabbit vs CS-1. Serum 2	
526	0.11	Severe
555	0.20	Severe
553	0.35	Fatal, 3 min
542	0.40	Fatal, 5 min
535	0.60	Fatal, 4 min
	Rabbit vs CS-1(Alum). Serum 3	
541	0.60	Slight
546	0.80	Moderate
543	1.00	Moderate
556	1.20	Moderate
530	2.00	Fatal, 3 min

* MSD = minimal passively sensitizing dose.

† Ml of original rabbit-antisera.

After an interval of 2 days, 3 additional intra-abdominal injections of 0.25 ml each were administered at 1-day intervals. A final 0.5-ml dose was injected subcutaneously after another interval of 2 days. For preparation of antisera nos. 12C, 13C, 16C, and 17C the animals were bled on the tenth day after the final injection of antigen.

Precipitin-tests

Precipitin-tests were conducted with the antisera as described in the first series except that twofold serial dilutions of the antigen were employed. The results are recorded in table 2.

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Precipitin-reactions of antiserum 15A, prepared from blood collected 2 days after the final injection of ovalbumin, and antiserum 15B, prepared from blood drawn 8 days after the final injection, were essentially the same. But the re-

TABLE 2

Precipitin-reactions of antisera

ANTISERUM NO.	ANTIGEN-DILUTION 1:															Normal-rabbit-serum control		
	25	50	100	200	400	800	1,600	3,200	6,400	12,800	25,600	51,200	102,400	204,800	409,600	25	50	100
	Rabbit vs ovalbumin sera																	
14B	0	±	1+	2+	3+	4+	6+	5+	4+	3+	2+	1+	1+	±	0	0		
14D	1+	1+	1+	1+	1+	1+	1+	2+	3+	4+	3+	2+	1+	±	0	0	0	0
15A	0	tr	tr	±	2+	3+	4+	4+	4+	3+	2+	1+	1+	±	0			0
15B	0	±	±	1+	2+	3+	4+	5+	3+	2+	1+	1+	1+	±	0			
15D	0	0	0	0	0	0	±	±	±	±	1+	2+	1+	±	0			
	Rabbit vs CS-1A sera															Normal-rabbit-serum control		
12A	0	0	0	0	0	0	0	0	0	0	0					0	0	0
12B	0	0	0	0	0	0	0	0	0	0	0					0	0	0
12C	0	0	0	0	0	0	tr	±	±	1+	1+	±	0	0	0	0	0	0
13B	0	±	1+	1+	2+	3+	4+	4+	3+	2+	1+	1+	±	0	0	0	0	0
13C	1+	1+	1+	1+	1+	2+	3+	4+	4+	3+	2+	1+	0	0	0			
	Rabbit vs CB-1A sera															Normal-rabbit-serum control		
16B	0	tr	tr	0	0	0	0	0	0	tr	±	±	0					
16C	tr	1+	1+	±	±	±	tr	tr	1+	1+	1+	±	0	0	0	tr	tr	0
17A	±	±	tr	0	0	0	0	0	0	0	0	0				±	0	0
17B	±	±	tr	0	0	0	0	0	tr	±	0	0	0					
17C	1+	1+	1+	1+	1+	1+	1+	1+	2+	2+	1+	±	0	0	0	tr	r	0

tr = barely perceptible trace.
A = Sera drawn 2 days after last injection of 1st course of sensitization.
B = Sera drawn 8 days after last injection of 1st course of sensitization.
C = Sera drawn 10 days after last injection of 2d course of sensitization.
D = Sera drawn 47 days after last injection of 1st course of sensitization.

actions of antiserum 15D, collected after the 47-day period, were significantly weaker and the range of optimal precipitation was shifted toward the higher dilutions of antigen. Optimal precipitation of antiserum 14B occurred at antigen dilution 1:1600 and the optimal reaction of antiserum 14D was at antigen dilution

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1:12,800. All ovalbumin-antisera titrated out to a maximal antigen-dilution of 1:204,800.

No precipitin for CS-1A was detected in antisera collected from rabbit no. 12 after a 2-day or an 8-day incubational period. But antisera from the same rabbit gave slight precipitation in the higher range of antigen-dilutions after the second course of sensitizing injections.

Strong reactions were observed in antisera for CS-1A collected from rabbit no. 13 after the 8-day incubational period and again after the second course of sensitizing injections.

Only doubtful precipitin-reactions were obtained with CB-1A antisera collected from rabbit no. 17 after the 2-day and the 8-day incubational periods. Antiserum 16B (8-day incubational period) likewise showed only slight precipitative

TABLE 3

Determination of MSD of stored rabbit-antisera by passive transfer to guinea-pigs

ANIMAL NO.	SENSITIZING DOSE	RESULT OF CHALLENGING INJECTION
	Rabbit vs ovalbumin. Serum 1	
	ml	
5552	0.01	Doubtful
5585	0.02	Fatal, 3 min
5599	0.04	Severe
5603	0.06	Fatal, 3 min
5608	0.08	Fatal, 3 min
5620	0.10	Fatal, 4 min
	Rabbit vs CS-1. Serum 2	
5592	0.05	None
5596	0.10	Delayed moderate
5600	0.20	Moderate
5604	0.40	Fatal, 3 min
5617	0.60	Fatal, 3 min
5621	0.80	Fatal, 2½ min

activity. Antisera from both rabbits nos. 16 and 17 showed evidence of increased precipitin-concentration as the result of the second course of sensitizing injections.

Passive-transfer experiments

The MSD of antisera for ovalbumin, CS-1A, and CB-1A were determined by the technic illustrated in table 1.

The MSD of the 2 ovalbumin-antisera from rabbit no. 14 were 0.005 ml for the 8-day incubational period and 0.06 ml for the 47-day period. For the 3 ovalbumin-antisera from rabbit no. 15 the MSD were, respectively, 0.02 ml, 0.01 ml, and 1.60 ml for the 2-day, 8-day, and 47-day incubational periods.

The MSD of the 3 CS-1A antisera from rabbit no. 12, collected after incubational periods of 2 days and 8 days and after resensitization, were >1.3 ml, >1.6 ml, and 0.32 ml, respectively. The MSD of the 2 CS-1A antisera from rabbit no.

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13, prepared after the 8-day incubational period and after resensitization, were the same, 0.02 ml.

The MSD of the 2 CB-1A antisera from rabbit no. 16 were 0.2 ml for the 8-day incubational period, and 0.04 ml after the second course of injections. The MSD of the 3 CB-1A antisera from rabbit no. 17 were 0.8 ml after the 2-day incubational period, 0.10 ml after the 8-day period, and 0.08 ml after the second course of injections.

Effect of storage on the antibody-content of rabbit antisera

When the second series of experiments were begun the antisera remaining from the first series were still in storage at 6 C, and appeared to be unchanged. It was of interest, therefore, to determine the potency of the sensitizing antibodies in the sera after refrigerated storage for 4 years and 9 months. Data for determination of the MSD of the antisera for ovalbumin (serum 1) and for CS-1 (serum 2) are presented in table 3. These results show that the capacity of the stored antisera to sensitize guniea-pigs fatally had remained essentially unchanged. Walzer and Grove (4) reported that neither the precipitin titer nor the sensitizing capacity of carbolized antisera changed appreciably during storage for one and a half years. Our results confirm their report with respect to the sensitizing capacity of stored antisera and extend the period of observation to nearly five years.

DISCUSSION

The passive-transfer technic was more sensitive than the precipitin-method for detecting antibodies in antisera. This fact has been observed by others (6). Moreover, the data showed that there was a definite parallelism between the potency of antiserum to induce passive anaphylactic sensitization in guinea-pigs and the capacity of the serum to exhibit the precipitin-reaction. Thus, antisera that induced fatal anaphylactic sensitization in guinea-pigs with dosages of 0.08 ml or less, showed moderate to strong precipitin-reactions. Antisera for CS-1A or for CB-1A with MSD ranging from 0.1 to 0.8 ml showed only weak or doubtful precipitation, and antisera for CS-1A with MSD greater than 1.3 ml gave no visible precipitin reactions. One exception to this generalization was observed. The MSD of ovalbumin-antiserum 15D was 1.6 ml. The capacity of this antiserum to give the precipitin-reaction may be related to the effect of molecular size of the antigen. Owing to its larger molecular size ovalbumin would be expected to yield visible antigen-antibody aggregates in weak antisera more readily than CS-1A or CB-1A. The observed parallelism between the precipitating capacities of antisera and capacities to induce passive anaphylaxis are in essential agreement with the original observations of Doerr and Russ (7).

From the foregoing results, it may be concluded that the allergenic fractions, CS-1A from cottonseed and CB-1A from castor beans, are less uniformly effective than ovalbumin in stimulating antibody-formation in rabbits. The allergenic fractions do not lack the capacity of precipitating with specific antibody but precipitin-reactions with these fractions require a higher concentration of antibody in the sera than is required with ovalbumin.

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There was evidence that antisera collected after the 8-day incubational period contained a higher effective concentration of sensitizing antibodies than those collected after the 2-day period. Thus, data were collected on antisera from 3 rabbits following both the 2-day period and the 8-day period. The sensitizing capacity of ovalbumin antiserum 15B (8-day period) had increased twofold over antiserum 15A (2-day period). CB-1A antiserum 17B (8-day period) had increased 8-fold over antiserum 17A (2-day period). The sensitizing capacities of the two antisera from the third animal (rabbit no. 12) was not disclosed in either case within the range of dosage employed. These differences in antibody-content were not detectable in the precipitin-tests as conducted by the antigen-dilution method.

Walzer and Grove (4) concluded that the intravenous method of passive sensitization of guinea-pigs was more constant and effective than the intra-abdominal method. Most of their animals, however, were tested for sensitivity within 24 hours after administration of the sensitizing dose. For example, in one experiment 9 animals were sensitized by the intra-abdominal route. Only 2 of this group died in shock and these 2 were challenged 48 and 72 hours, respectively, after administration of the sensitizing serum. The lowest MSD of ovalbumin antisera recorded by Walzer and Grove was 0.045 ml when the serum was administered by the intravenous route. In our experiments the MSD of ovalbumin antisera from rabbits sensitized by the Walzer-Grove method ranged from 0.005 to 0.04 ml when the serum was administered by the intra-abdominal route. Accordingly, these observations indicate that if the challenging injection is given after an interval of 48 hours the intra-abdominal sensitizing injection is as reliable as the intravenous method.

SUMMARY

The allergenic proteic-polysaccharidic fractions, CS-1A from cottonseed and CB-1A from castor beans, were precipitinogenic when administered to rabbits. The allergenic fractions were less uniformly effective than ovalbumin in stimulating precipitin-formation.

The passive-transfer technic provided a more sensitive method for detecting antibodies in sera than the precipitin-method.

The passive-transfer sensitizing capacity of antisera, preserved with 0.25 per cent phenol and stored at 6 C, remained essentially unchanged for nearly five years.

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