

LOCALIZATION OF BLOCKING ANTIBODY IN SERA OF RAGWEED-SENSITIVE INDIVIDUALS BY STARCH ELECTROPHORESIS

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IN A recent report from this laboratory¹ it was shown that sera of ragweed-sensitive persons could be separated into electrophoretically distinct serum fractions by starch electrophoresis, and that the skin-sensitizing activity was associated with the gamma₁ and/or gamma₂ globulins. All other serum fractions were found to be devoid of reaginic activity.

In continuation of our studies on antibody-like factors in allergic sera, starch electrophoresis was used in the present investigation for the localization of blocking antibodies in the sera of treated allergic persons. Since the initiation of the present work, two independent reports^{2, 3} have been published recently on the localization of the blocking antibody to ragweed pollen. Cooke and associates,² using the combined methods of ammonium sulfate fractionation, electrophoresis-convection, and free electrophoresis with the aid of the preparative macro-Tiselius cell, and Loveless and Cann,³ using electrophoresis-convection, were able to demonstrate that the bulk of the blocking antibody was associated with gamma globulins. However, the possibility of some blocking activity being associated with other serum proteins could not be unequivocally excluded. The results of our study, presented in this article, indicate that blocking antibody is associated exclusively with gamma globulins. .

EXPERIMENTAL

Blocking sera were obtained from six ragweed-sensitive patients who had been given an intensive course of injections of whole ragweed pollen extract for a period of two to three months prior to bleeding. About 100 ml. of blood was collected from each patient. The blood was processed in the usual way.¹

Zone Electrophoresis.—Since the present study constitutes only a preliminary survey as regards the localization of the blocking antibody, the sera were separated into pooled "peak fractions,"¹ namely, into the clearly delineated albumin, and α_1 -, α_2 -, β -, γ_1 -, and γ_2 -globulin fractions, and not into the different serum subfractions (40 to 60 eluates) as was done in the study of the

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These studies were supported by a grant from the Department of National Health and Welfare, Canada, and from the Charles E. Frost Company, Montreal, Eli Lilly and Company, Indianapolis, and Frank W. Horner Company, Montreal.

Received for publication Oct. 8, 1956.

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skin-sensitizing antibody. The procedure used was otherwise identical in all respects to that described previously.¹ The pooled eluates, constituting the different, electrophoretically distinct, serum fractions were individually Seitz filtered into sterile Visking tubings and concentrated by pervaporation to final volumes of about 6 to 8 ml. Pervaporation was alternated with dialysis against 0.9 per cent saline solution in order to avoid excessive concentration of salts in the Visking tubings. For the same reason, in some experiments the sterile contents of the Visking tubing were dialyzed against distilled water or tap water prior to pervaporation. In the latter cases pervaporation was performed without interruption until the desired final concentration was reached. The concentrated serum fractions to be skin tested were then dialyzed against saline for about twenty-four hours and transferred into sterile vials through Swinny-Seitz filters. The sterile fractions and whole sera were stored at 4° C.

Tiselius Electrophoresis.—This was used for the analysis of the whole sera. The method as applied in this laboratory was fully described previously.¹ The individual pooled "peak fractions" were not recharacterized by Tiselius electrophoresis, since it was shown earlier¹ that the designation of the various serum components on the basis of the protein distribution curve obtained by starch electrophoresis corresponded closely to that commonly accepted for free electrophoresis.

Detection and Titration of Blocking Antibody.—The method used in this study was essentially similar to that described by Loveless^{3, 4} and by Cooke and associates.² It depends primarily on the ability of blocking antibody to combine or inactivate in vitro a known amount of the corresponding allergen. In this method a known amount of reagin is used as internal indicator. The method consisted of two stages: (1) titration of the reagin content in an aliquot of serum containing only skin-sensitizing antibody with a known amount of allergen and (2) titration of the blocking antibody. To avoid complications due to the presence of reagin, which is normally present in addition to blocking antibody in sera of treated allergic persons, the former was inactivated prior to the titration of blocking antibody in the whole sera and in the serum fractions by heating at 56° C. for periods of as long as six hours.

Stage 1: A reaginic serum, obtained from an untreated ragweed-allergic patient, was diluted with saline so that 1 ml. of the solution contained sufficient reagin to neutralize approximately 200 units of ragweed pollen.* This solution will be referred to hereafter as R. Six sites on the back of a normal nonsensitive person were injected with 0.05 ml. of solution R. Twenty-four hours later these sites were injected with 0.05 ml. of ragweed pollen extract of varying concentrations (for example, from 50 to 300 units per milliliter). Within twenty to forty minutes, typical wheals and flares developed at each site, the minimum reaction being given by the sites injected with the lowest

*One unit corresponds to 10⁻⁵ mg. nitrogen.

dose of ragweed pollen. However, no direct relationship was observed between the amount of ragweed injected and the extent of reaction.* On the third day all sites were injected with 0.05 ml. of a concentrated ragweed pollen extract (500 to 600 units per milliliter). The sites which had received previously (on the second day) amounts of ragweed in excess of that required for neutralizing the reagin content in 0.05 ml. of solution R did not give any reactions. The sites which had received smaller amounts of the allergen than that required for neutralization gave positive reactions. The true neutralizing dose was considered to correspond to the average between the lowest dose of ragweed injected on the second day, which was just short of neutralizing all the reagin, and the next higher dose which had reacted with all the reagin. The scheme of a typical experiment for the titration of reagin in solution R is given in Table I. Additional control sites were injected on the first day with saline and on the third day with the ragweed solution containing 500 units per milliliter. The sites were marked with carbolfuchsin to facilitate their recognition. All tests were done in triplicate on the same subject. Each experiment was repeated on several subjects.

TABLE I

		SITES					
		1	2	3	4	5	6
First day	Sensitization with R*	0.05 ml.	0.05 ml.	0.05 ml.	0.05 ml.	0.05 ml.	0.05 ml.
Second day	Injection of allergen†	50 u./ml.	100 u./ml.	150 u./ml.	200 u./ml.	250 u./ml.	300 u./ml.
Third day	Injection of allergen†	500 u./ml.	500 u./ml.	500 u./ml.	500 u./ml.	500 u./ml.	500 u./ml.

*R represents reaginic serum diluted 1:100.

†0.05 ml. used for each injection, 1 u. = 10^{-5} mg. nitrogen.

Stage 2: For the detection of blocking antibody, the following procedure was used: An aliquot of the whole serum or serum fraction suspected of containing blocking antibody was mixed in a sterile vial with a calculated amount of allergen (An) and incubated at 4° C. for twenty-four hours (mixture An-B).† An appropriate volume of the reaginic serum‡ used in Stage 1, corresponding to that which would be neutralized by An units of the allergen, was then added to the mixture "An-B" and the vial was allowed to stand at room temperature for about one hour. Subsequently, 0.05 ml. of the combined mixture was injected into the skin of a nonallergic person. Control sites were injected with a mixture containing the same amounts of reagin and ragweed as in the test mixture, the only difference being that the blocking serum was replaced by saline. Some of the sites gave immediate reactions which were ignored since they could not be graded. Twenty-four hours later both test and control sites were injected with excess allergen. The control sites gave no reactions, since there was no free reagin left to react with the additional

*This problem is fully discussed elsewhere.⁵

†The solutions were filtered through a Swinny-Seitz filter.

‡The same reaginic serum was used in all blocking experiments.

allergen injected on the second day. On the other hand, sites containing blocking antibody gave a wheal and erythema, thus demonstrating that some of the allergen An had been "blocked" during incubation and that reagin was still available for further reaction with the allergen.

For the titration of blocking antibody, a series of mixtures containing the blocking serum in halving dilutions and constant amounts of reagin and ragweed (the latter two in neutralizing amounts with respect to each other) were made up as described above. Then 0.05 ml. of these mixtures was injected into the skin of a test subject. After twenty-four hours all sites were injected with excess allergen. The sites sensitized with mixtures containing the highest amount of blocking antibody gave the strongest reaction. The other sites, containing blocking antibody in decreasing amounts, gave smaller and graded reactions. The blocking titer was given by the $(\frac{1}{2})^n$ -th dilution of the blocking serum (where n represents the number of half-dilutions) which still gave a detectable reaction. Thus, using the same amount of reagin and the corresponding neutralizing dose of allergen, the degree of blocking ability could be established for a series of blocking sera. All tests were done in triplicate and on more than one subject. A typical experiment for the titration of blocking antibody is illustrated in Table II.

TABLE II. PROCEDURE FOR TITRATION OF BLOCKING ANTIBODY

	TEST MIXTURES							
	1	2	3	4	5	6	7	8
2 ml. of blocking serum in dilutions	1:1	1:2	1:4	1:8	1:16	1:32	1:64	0 ml.
Volume of saline	0	0	0	0	0	0	0	2 ml.
Allergen solution*	1 ml.	1 ml.	1 ml.	1 ml.	1 ml.	1 ml.	1 ml.	1 ml.
All mixtures incubated at 4° C. for 24 hours, followed by addition of reagin solution.								
Reagin solution*	1 ml.	1 ml.	1 ml.	1 ml.	1 ml.	1 ml.	1 ml.	1 ml.
After incubation for 1 hour at room temperature, 0.05 ml. of each mixture was used for Prausnitz-Küstner test.								

*The allergen and reagin solutions were used in neutralizing concentrations with respect to each other, as determined in titration experiments. Since both solutions were diluted four-fold in the preparation of these test mixtures, their concentrations were four times as high as those used in titration experiments.

The allergen solutions used for making up the mixture "An-B" and for challenging the sensitized sites consisted of a water-soluble ragweed pollen extract (WSR) in preliminary experiments. Since it caused some irritation and did not give consistently reproducible results, it was replaced by a ragweed pollen fraction, named AA₁+Pt* (prepared by zone electrophoresis using filter paper as a supporting medium) which gave well-defined reactions without irritation. The justification for using AA₁+Pt instead of WSR was afforded by the fact that the same blocking titer was obtained with either allergenic solution. The sera separated by starch electrophoresis were heated just prior to the titration of the blocking antibody. The concentrated fractions

*We are indebted to Mr. M. Richter of this laboratory for the AA₁+Pt ragweed pollen fraction. The preparation of this fraction is described elsewhere.⁶

were tested for reaginic content before determination of their blocking titer. The fractions containing reagin were heated at 56° C. for a period not longer than necessary to inactivate reagin.

RESULTS AND DISCUSSION

The results of free electrophoresis for the six sera studied are given in Table III, together with our values for normal sera. The extent of reaginic activity of the six sera, as measured by the degree of dilution which still gave a positive skin test, is represented in Table IV. It is interesting to note that

TABLE III

EXPERI- MENT NO.	SERUM	T.P.*	ALB.		α_1		α_2		β		γ	
		GM./ 100 ML.	PER CENT	GM./ 100 ML.	PER CENT	GM./ 100 ML.	PER CENT	GM./ 100 ML.	PER CENT	GM./ 100 ML.	PER CENT	GM./ 100 ML.
E 1064	A	7.20	63.17	4.55	3.49	0.25	7.56	0.54	13.27	0.95	12.50	0.90
E 996	Sh	7.32	61.89	4.53	4.93	0.36	5.54	0.40	14.09	1.03	13.55	0.99
E 947	P	6.84	52.70	3.60	3.38	0.23	7.97	0.54	13.58	0.93	22.36	1.53
E 1266	W I	7.98	61.22	4.88	4.61	0.37	8.37	0.67	12.06	0.96	13.73	1.09
E 1414	W II	7.32	62.97	4.61	4.62	0.34	6.65	0.47	14.77	1.08	10.99	0.80
E 1412	D II	7.26	51.86	3.76	5.50	0.40	11.78	0.85	15.58	1.13	15.27	1.11

NORMAL SERA

	T.P.	ALB.		α_1		α_2		β		γ	
	GM./ 100 ML.	PER CENT	GM./ 100 ML.	PER CENT	GM./ 100 ML.	PER CENT	GM./ 100 ML.	PER CENT	GM./ 100 ML.	PER CENT	GM./ 100 ML.

22 Males

Average	7.93	59.46	4.73	4.68	0.36	8.60	0.69	12.92	1.06	14.33	1.08
S.D.	±0.56	±3.16	±0.31	±1.81	±0.06	±1.43	±0.16	±1.27	±0.12	±22.46	±0.19

14 Females

Average	7.92	58.40	4.69	5.17	0.42	9.81	0.72	12.42	1.00	14.18	1.08
S.D.	±0.71	±2.31	±0.39	±0.73	±0.07	±0.98	±0.13	±1.18	±0.11	±1.76	±0.17

*T.P. refers to total protein, determined refractometrically.

the first, serum A, did not give a positive Prausnitz-Küstner reaction, although a strongly positive skin test was given by the patient who also had clinical symptoms characteristic of ragweed hay fever. The other five sera gave strongly positive reactions in normal skin.

TABLE IV

SERUM	REAGIN TITER	BLOCKING TITER
A	0	1:4
Sh	1:1,000	1:4
P	1:10,000	1:8
W I	1:1,000	1:32
W II	1:1,000	1:16
D II	1:1,000	1:64

All six sera were shown to contain blocking activity after treatment. Their blocking titers are given in Table IV. The first two sera, A and Sh, exhibited only weak blocking ability, which could not be detected in dilutions higher than 1:4. The other four sera had reasonably good blocking titers.

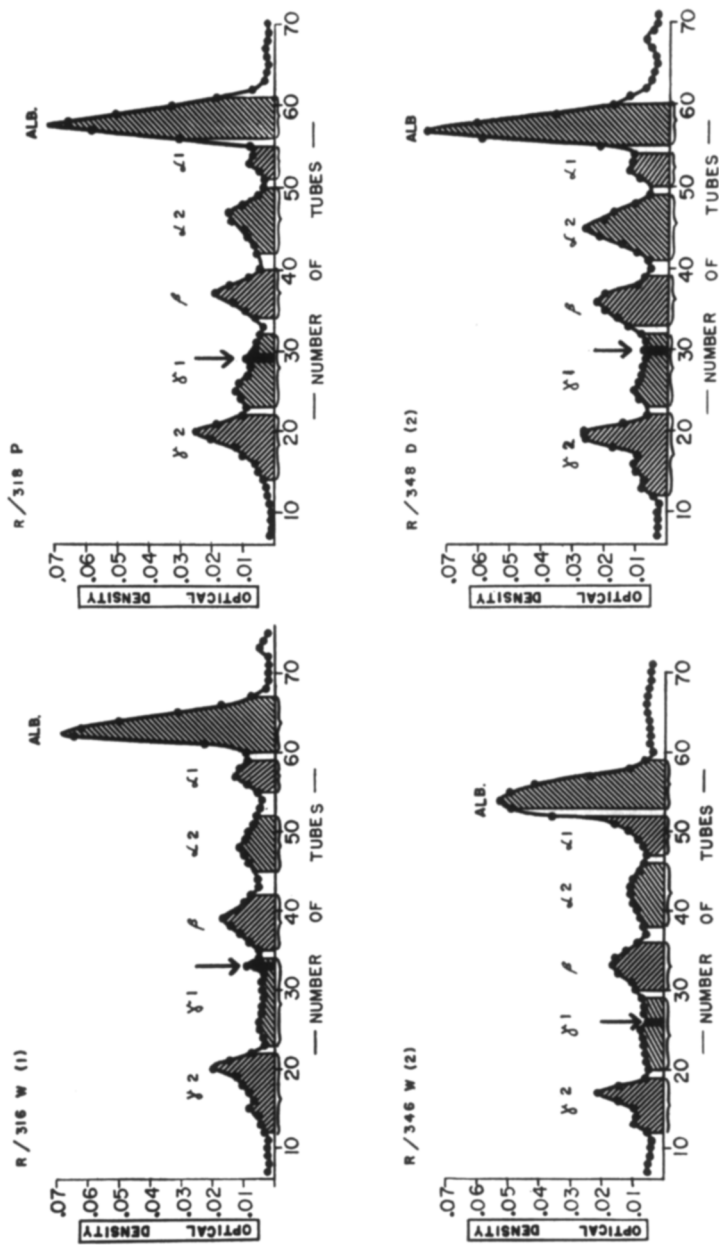


Fig. 1.—Separation of blocking sera.

The electrophoretic separation of four sera by starch electrophoresis is illustrated in Fig. 1. Since the primary aim in the present study was to develop a method for the demonstration of blocking antibody in the serum fraction(s) with which it is associated, the starch blocks were divided into only five or six large fractions without eliminating the possible contamination due to serum proteins present in the interzonal regions. Blocking activity could not be clearly demonstrated in any of the fractions of the two weakly blocking sera, A and Sh. In all the other four sera, however, blocking activity was found to be associated with the γ_2 globulins. In addition, in one of the four sera, namely, D II, some blocking activity was also detected in the γ_1 globulins. All other serum fractions were found to be completely devoid of any blocking activity. Our results confirm the previous findings of Cooke and associates² and Loveless and Cann³ and demonstrate that blocking activity in sera of ragweed-sensitive patients is confined to γ -globulins only, mainly to the slow γ_2 -globulins.

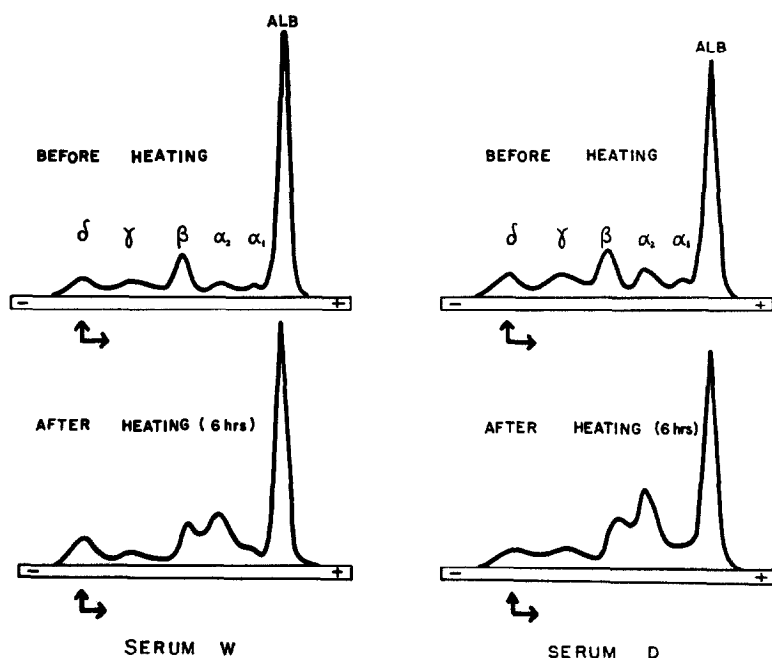


Fig. 2.—Effect of heating on human serum (56 degrees).

In sera P, Sh, and W I, reaginic activity was found to be associated only with the γ_1 -globulins. These findings are similar, to some extent, to those of Kuhns,⁷ who has shown that skin-sensitizing antibodies and blocking non-precipitating antibodies to diphtheria toxin are found in the γ_1 - and γ_2 -globulins, respectively.

In this connection, it is also worth mentioning that Cooke and associates,² using ammonium sulfate fractionation, showed that the skin-sensitizing and blocking antibodies to ragweed were associated with two different fractions, isolated by precipitation at 55 per cent and 33.3 per cent ammonium sulfate,

respectively. In our own experience, serum proteins precipitated at 33.3 per cent ammonium sulfate contain mainly γ_2 -globulins, while the additional fraction precipitated at 55 per cent ammonium sulfate contains the faster moving α -, β -, and γ_1 -globulins in addition to small amounts of γ_2 -globulins.

The reagin present in the sera to be fractionated was inactivated by heating only after separation on the starch blocks, since, as one would expect,⁸ heating at 56° C. for periods as long as six hours caused considerable alteration of some serum proteins, as demonstrated by electrophoretic analysis. This is illustrated in Fig. 2. As can be seen, the β -globulins of the whole sera were affected, on heating, to a larger extent than any of the other serum proteins, the net effect manifesting itself by an increase in mobility of the altered proteins. The sera remained clear after heating for periods of as long as six hours. Electrophoretic analysis of sera heated for varying shorter times showed that the alteration of the β -globulins was a gradual process. These findings confirm other results obtained in this laboratory,⁹ suggesting that the β -globulins, in all probability the β -lipoproteins, are the most labile components of human serum, at least as determined by electrophoresis.

The complexity of the test for blocking antibody is self-evident. The main source of error in determining the blocking titer lies in establishing the correct dose of allergen necessary to neutralize the reagin used as the indicator of the titration. Incorporation in the mixture "An-B" of larger amounts of allergen than necessary will lead to an erroneously low blocking titer or to complete failure in detecting any blocking antibody. Conversely, addition of lower quantities of allergen will give an apparently higher blocking titer than the true one. However, it ought to be stressed that, in spite of these difficulties, the results obtained with any given series of test mixtures were independent of the normal skin used. Attempts at increasing the sensitivity of the test for blocking antibody are being made and will be reported elsewhere.

SUMMARY

Using starch electrophoresis, blocking activity of four sera of treated ragweed-sensitive patients was demonstrated to reside mainly in the slow-moving gamma globulins. No blocking antibodies were detected in other serum fractions. The method of detection and titration of blocking antibodies is described in detail.

We should like to acknowledge the skillful technical assistance of Mr. P. L. Rojowski, Mr. S. Kennedy, and Mrs. A. Elovic in these experiments.

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