

Physicochemical properties of reaginic antibody

I. Association of reaginic activity with an immunoglobulin other than γ A- or γ G-globulin

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Two reaginic sera from atopic patients were fractionated by chromatography on DEAE cellulose columns and by gel filtration through Sephadex G-200 columns. The skin-sensitizing activity in the chromatographic fractions did not parallel the concentration of either γ A- or γ G-globulin. Gamma G and γ A-globulins in the reagin-containing fractions were precipitated with rabbit antibodies which are specific for either γ G- or γ A-globulin. Essentially all skin-sensitizing activity in the fractions remained in the supernatant after the precipitation of both immunoglobulins. The reaginic activity in the supernatant was coprecipitated with γ G-globulin-anti-light chain antibody complexes. These results indicated that the skin-sensitizing antibody was associated with neither γ G- nor γ A-globulin but with a unique protein having light chain(s) of immunoglobulins. It was also found that 0.001 to 0.002 μ g N of this protein was sufficient for the passive sensitization of normal human skin for positive P-K reactions. The possible carrier of reaginic activity was discussed.

The physicochemical properties of human reaginic antibody have been studied by many investigators. Electrophoretically, the antibody has been detected in the slow β - to fast γ -globulin.^{1, 2} The Cohn low temperature ethanol fractionation procedure concentrated reaginic activity in Fraction III which is predominantly composed of β -globulin.³ Recently, Heremans and Vaerman⁴ have reported that the γ A-globulin fraction of the sera of allergic patients showed Prausnitz-Küstner sensitization activities of the same order of magnitude as the corresponding whole sera, whereas the γ G-globulin concentrations never exceeded 1 per cent of that in serum. Fireman and associates⁵ reported that the reaginic

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antibody in human sera was precipitated by the addition of sheep anti- γ A-globulin antibody, but not with anti- γ G-globulin, indicating that the reaginic antibody is associated with γ A-globulin. Our previous studies also supported this idea, through the demonstration that normal γ A-globulin, but not γ G- or γ M-globulin, blocked passive sensitization of normal human skin with reagin.⁶ Yagi and associates⁷ detected γ A-, γ M-, and γ G-globulin antibodies to ragweed pollen extract in sera by radioimmuno-electrophoresis. Augustin and associates,⁸ as well as Vaerman and co-workers,⁹ demonstrated reaginic activity in pure γ A-globulin preparations from reaginic sera. All of these findings support the view that reaginic antibody is γ A-globulin. However, Loveless¹⁰ has demonstrated reaginic activity in a serum sample which lacked a detectable amount of γ A-globulin by immunoelectrophoresis. In the course of our studies⁹ on the γ A-globulin fraction of reaginic sera, reaginic activity was detected in both γ G- and γ A-globulin fractions of one of the serum samples. These findings suggested to us the idea that reaginic antibody is not necessarily γ A-globulin. The present investigation was undertaken to study this possibility. Two serum samples having high reaginic activity were fractionated by column chromatography. Comparisons were made between the relative concentrations of γ G- and γ A-globulin and reaginic activity in the fractions. The fractions containing high reaginic activity were absorbed with rabbit antisera specifically for γ G- or γ A-globulin. The results suggest that the reaginic antibody in two reaginic sera is not γ A-globulin.

MATERIALS AND METHODS

Reaginic sera

The high titer reaginic sera were obtained from an egg-sensitive patient and a ragweed-sensitive patient.* Both sera were kept in the frozen state at -40° C. until used. In Prausnitz-Küstner (P-K) reactions (see below), as high as 1:2,000 to 1:5,000 dilutions of the sera sensitized normal skin for definite erythema-wheal reactions.

Normal human immunoglobulins and γ G-globulin fragments

Human γ G-globulin was isolated from Fraction II† by chromatography on a diethylaminoethyl (DEAE) cellulose column. The protein was dissolved in 0.01M phosphate buffer (pH 8.0) and applied to a DEAE cellulose column which was equilibrated with the buffer. The protein fractions eluted with the same phosphate buffer were pooled and concentrated by negative pressure filtration. Gamma A globulin was purified from normal human serum by zinc precipitation following the procedure of Vaerman and associates.¹¹

Gamma G globulin was digested with crystalline papain (2 $\times$ recrystallized)† by Porter's method¹² without modification. F_c and F_{ab} fragments in the digest were isolated by starch block electrophoresis with barbital buffer (pH 8.6, 0.1 μ), as described by Olins and Edelman.¹³ Eluates from each centimeter segment were tested by immunodiffusion against an anti-human γ G-globulin serum which contained antibodies for both heavy and light chains. The fast and slow migrating

*The latter serum was a gift from Dr. Maxwell Richter, Montreal, Canada.

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fractions which gave a single precipitin band with the antiserum were pooled separately.

Antisera specific for human immunoglobulins

Goat antiserum against whole human serum and that specific for γ A-globulin were purchased* and rabbit antisera specific for γ A-, γ G-globulin, or F_{ab} fragment were prepared. Five milligrams of γ A-globulin included in complete Freund adjuvant† was injected into the footpads. Two weeks later, 5 mg. of the same protein in complete Freund adjuvant was injected intramuscularly and the rabbits bled two weeks after the last immunization. Anti- γ G sera were obtained by the immunization of rabbits with increasing amounts of alum-precipitated γ G-globulin for four weeks. They were bled one week after the last immunization. The γ -globulin fraction of the antisera was obtained by precipitation with ammonium sulfate at one-third saturation and pH 7.8. Cross-reacting antibody in the γ -globulin fraction of anti- γ A serum was removed by absorption with γ G-globulin at the equivalence ratio. The antibody specific for γ G-globulin was obtained by the absorption of the anti- γ G-globulin with F_{ab} fragment. The γ -globulin fraction of the same serum was absorbed with F_c fragment to prepare the antibody specific for light chains. The γ -globulin fractions of the specific antisera were further purified by chromatography on a DEAE cellulose column. The protein eluted from the column with 0.01M phosphate buffer (pH 8.0) was concentrated. Specificity of antibodies present in rabbit anti- γ G, anti- γ A and anti-light chain antibody preparations was tested by immunodiffusion, using human serum, purified immunoglobulins, and fragments of γ G-globulin. The results are shown in Fig. 1. Rabbit and goat antisera specific for γ A-globulin gave a single precipitin band of identical specificity with normal human serum. The concentration of specific antibody in the final preparations was measured by quantitative precipitin reactions¹⁴ with the use of purified γ G- and γ A-globulins as antigens. Precipitating antibody nitrogen in the anti- γ G, anti- γ A, and anti-light chain preparations was 320 μ g, 240 μ g, and 153 μ g per milliliter, respectively.

Column chromatography

Reaginic sera were fractionated by chromatography on DEAE cellulose columns and by gel filtration. The protein solution was applied to a DEAE column (2 by 45 cm.) which had been equilibrated with 0.005M phosphate buffer of pH 8.0. Protein was eluted stepwise with phosphate buffers (pH 8.0) of 0.005M to 0.15M. Five milliliter fractions were collected and absorption at 280 $m\mu$ was determined.

Gel filtration was carried out using Sephadex G-200.‡ A 5 to 6 milliliter sample was applied to a Sephadex column (2 by 110 cm.) and eluted with borate buffered saline (pH 8.0). In some experiments, 15 ml. samples were applied to 3.5 by 120 cm. columns.

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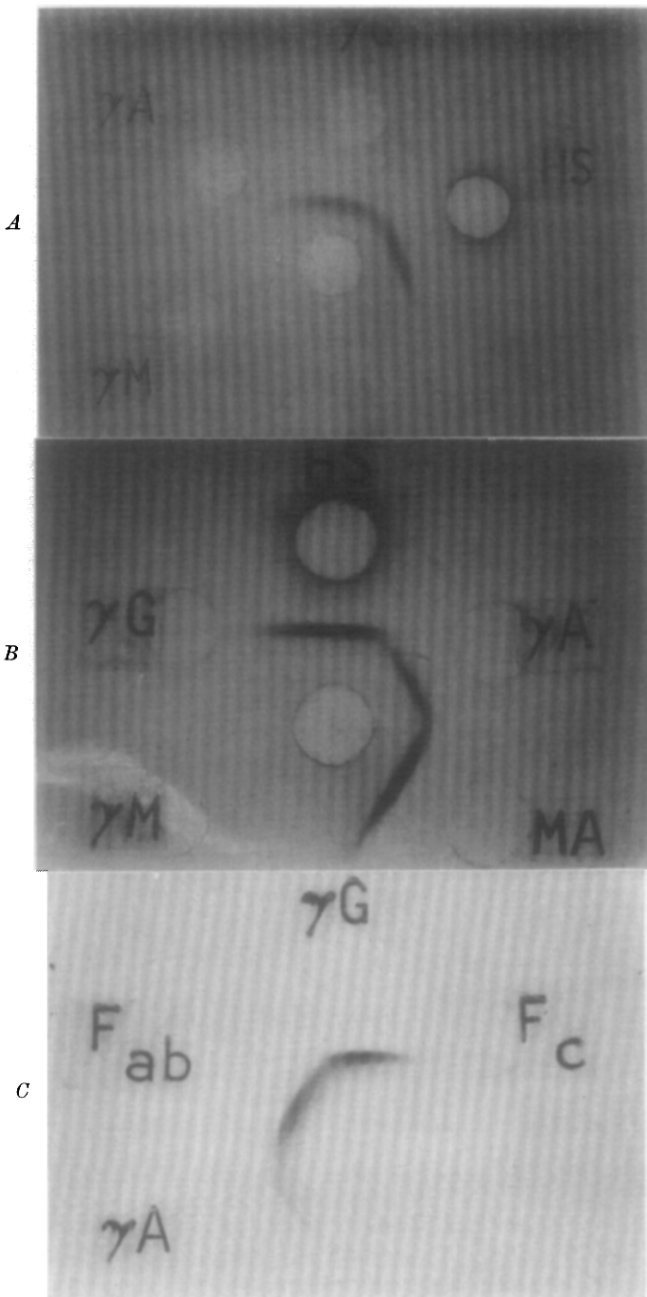


Fig. 1, A-C

Immunodiffusion analyses of rabbit antibody preparations. Center wells were filled with anti- γG (A), anti- γA (B) and anti-light chain (C) antibody preparations. Peripheral wells were filled with human serum (HS), γG -, γA -, γM -globulins, and/or F_{ab} and F_c fragments. MA represents γA myeloma protein.

Radioiodination

A reagin-containing fraction of the ragweed-sensitive patient's serum was labeled with I^{131} by the method of Day and co-workers.¹⁵ Radioactive iodine in sodium iodide was obtained* and radioactivity measurements were made with a well-shaped scintillation counter.

Immunodiffusion

Double diffusion in agar plates was carried out by the method of Ouchterlony.¹⁶ Immunoelectrophoresis was performed on microscope slides, following the modification of Scheidegger.¹⁷ The plates were incubated at room temperature for 48 hours, washed, and the precipitin bands stained with azocarmine. The concentrations of γ G- and γ A-globulins in the chromatographic fractions of reaginic sera were determined with immunoplates following the method of Mancini and associates.¹⁸ The rabbit antibody specific for either γ G- or γ A-globulin was included in agar plates. The final concentration of the precipitating antibody in agar was 25 μ g N per milliliter. The wells in the plates were filled with fractions of reaginic sera. As standards, serial dilutions (6.5 to 200 μ g N per milliliter) of γ G- or γ A-globulin solution were tested simultaneously. After four days at room temperature, the precipitating areas around the wells were measured under magnification. The concentration of each protein in the fractions was estimated from standard curves.

Prausnitz-Küstner reactions

The tests were carried out on nonallergic individuals. One-twentieth milliliter of test samples was injected, intracutaneously. Twenty-four hours later, the sites received 0.02 ml. of 0.1 per cent egg albumin (5 $\times$ recrystallized),[†] or of ragweed allergen extract[‡] containing 2 PN units. Erythema-wheal reactions, which appeared 15 to 20 minutes after the injection of allergen, were traced on a transparent plate which was applied to the area. When the reaginic activity of several samples was compared, all of the samples were tested in the same individuals in order to minimize individual differences in skin responses. The P-K tests for each sample were duplicated in two to three different normal individuals.

EXPERIMENTAL RESULTS

The serum of an egg-sensitive patient was dialyzed against 0.02M phosphate buffer (pH 6.0) for two days. Euglobulin was removed by centrifugation and pseudoglobulin in the supernatant was precipitated at one-half saturation of ammonium sulfate at pH 7.0. The precipitate was dissolved in the original volume of saline and reprecipitated twice in the same manner. The final precipitate was dissolved in borate buffered saline. After dialysis against the buffer, a trace amount of precipitate was removed by centrifugation at 20,000 r.p.m. for 30 minutes. The supernatant was then fractionated by gel filtration through

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a Sephadex G-200 column (3.5 by 120 cm.). As reported previously,^{5, 17, 20} three peaks were obtained and the γ A-globulin was eluted before the bulk of γ G-globulin in the second peak. The ascending portion of the second peak was pooled, concentrated, and dialyzed against 0.005M phosphate buffer. The solution was then applied to a DEAE cellulose column and eluted stepwise with 7 phosphate buffers (pH 8.0) increasing in molarity, i.e., 0.005, 0.01, 0.02, 0.035, 0.05, 0.08, and 0.15M buffers. The protein eluted with each buffer was pooled separately (Fractions I to VII), concentrated, and analyzed by immunoelectrophoresis against goat anti-human serum. Only one precipitin band in the γ -globulin region was obtained with Fractions I and II, whereas the other fractions gave two or more precipitin bands. The presence of γ G- and γ A-globulins in the fractions was studied by immunodiffusion against rabbit antisera specific for one of these proteins. Gamma G globulin was detected in all fractions, whereas γ A-globulin was detected in Fractions III to VII. The same results were obtained with goat anti-A globulin antibody. Neither Fractions I (330 μ g N per milliliter) nor II (170 μ g N per milliliter) gave a precipitin reaction with anti- γ A-antibody by interfacial ring tests in which as little as 1 μ g N per milliliter of normal γ A-globulin can be detected. Relative concentration of γ G- and γ A-globulins in the fractions was estimated by immunoplates which included rabbit antibody specific for either γ A- or γ G-globulin. Neither Fractions I nor II formed a precipitin ring in the plates containing anti- γ A-globulin. The relative concentrations of the globulins in the fractions are shown in Table I.

The skin-sensitizing activity of each fraction was tested by P-K reactions. Twofold dilutions of the fractions were injected intracutaneously into normal individuals and the skin sites were challenged with egg albumin 24 hours later. The results shown in Table I indicate that Fraction III has the maximum skin-sensitizing activity on a weight basis. The quantity of γ G- and γ A-globulins in the minimum skin-sensitizing dose of the fractions was calculated. It is evident that the reaginic activity of the fractions did not parallel the concentration of either γ A- or γ G-globulin (Table I).

Table I. *Skin-sensitizing activity and γ -globulin concentrations in chromatographic fractions of an egg-sensitive patient's serum*

Fractions	Phosphate buffer for elution* (mole/L.)	Relative concentration		Minimum skin-sensitizing dose		
		γ G (%)	γ A (%)	Total N† (μ g N)	γ G (μ g N)	γ A (μ g N)
I	0.005	100	0†	> 2	> 2	—
II	0.01	100	0†	0.04	0.04	< 0.0008
III	0.02	89	11	0.02	0.018	0.002
IV	0.035	33	35	0.04	0.012	0.014
V	0.05	32	27	0.2	0.064	0.054
VI	0.08	12	13	1.0	0.12	0.13
VII	0.15	8.5	7	2.0	0.17	0.14

*Stepwise elution from a DEAE cellulose column.

†The P-K reactions for each sample were carried out in two different normal individuals with comparable results.

‡No precipitin ring was formed on the immunoplate containing anti- γ A-globulin. The protein concentrations in the fractions were 150 to 300 μ g N per milliliter.

Since high reaginic activity was detected in Fractions II, III, and IV, these fractions were absorbed with rabbit antibody specific for γ G- or γ A-globulins. Equal volumes of the antibody preparations were added to appropriate dilutions of the fractions, so that the immunoglobulin/antibody ratio ranged from equivalence to slight antibody excess. Although Fraction II did not contain a detectable amount of γ A-globulin, anti- γ A-globulin was added to the fraction: Fraction II containing 30 μ g N was mixed with the amount of antibody which was sufficient to precipitate 20 μ g N of γ A-globulin at the equivalence point. All tubes were kept at 1 to 2° C. overnight and centrifuged to remove specific precipitates. The supernatants were tested for the presence of γ G- and γ A-globulins by immunodiffusion and by interfacial ring tests with the antisera used for absorption. No γ G-globulin or γ A-globulin was detected in the supernatant after absorption with homologous antibody. Skin-sensitizing activity of the supernatants was then tested by P-K reactions. Dilutions of the unabsorbed preparation were injected into the same normal individuals as controls. The results, shown in Table II, indicate that the precipitation of γ G-globulin from Fractions II, III, and IV has no effect on the skin-sensitizing activity. The precipitation of γ A-globulin in Fraction III, which had the highest skin-sensitizing activity, was not accompanied by any detectable decrease in the activity. After precipitation of γ A-globulin from Fraction IV, the skin-sensitizing activity decreased to half of that obtained with the original preparation. The addition of anti- γ A-globulin to Fraction II slightly decreased the skin-sensitizing activity, although no precipitate was formed between anti- γ A and this fraction. It is evident that most of the skin-sensitizing antibody in the fractions is not associated with either γ A- or γ G-globulin.

Table II. *Effect of absorption with anti- γ -globulin on skin-sensitizing activity in fractions of egg-sensitive serum**

Frac- tion†	Absorption with	Presence of‡		Dilution of fractions injected		
		γ G	γ A	10 × [0.35]§	50 × [0.07]	100 × [0.035]
II	anti- γ G	—	—	33 × 46 (15)	29 × 38 (8.5)	25 × 28 (8)
	anti- γ A	+	—	40 × 55 (15)	22 × 30 (7)	(—)
	—	+	—	45 × 56 (14)	20 × 22 (8)	16 × 20 (6.5)
III				30 × [0.25]§	300 × [0.025]	600 × [0.0125]
	anti- γ G	—	+	43 × 47 (13)	30 × 32 (8)	—
	anti- γ A	+	—	35 × 60 (12)	25 × 41 (7)	—
	—	+	+	45 × 52 (13)	32 × 39 (8)	—
IV				30 × [0.4]§	150 × [0.08]	300 × [0.04]
	anti- γ G	—	+	33 × 36 (12)	30 × 26 (7)	21 × 30 (7.0)
	anti- γ A	+	—	27 × 34 (10)	25 × 31 (7)	(—)
	—	+	+	34 × 35 (12)	26 × 34 (8)	22 × 24 (7.0)

*Numbers in this table represent the average diameters of erythema in millimeters; the numerals in parentheses represent the average diameter of the wheal in P-K reactions in 3 normal individuals.

†Chromatographic fractions (cf. Table I).

‡Tested by interfacial ring tests and by immunodiffusion.

§[] Total N [μ g] injected in the unabsorbed fraction.

The experiments were repeated with slight modifications, using a ragweed-sensitive serum. The globulin fraction of the serum was obtained by precipitation with one-half saturation of ammonium sulfate and was further fractionated by chromatography on a DEAE cellulose column. Stepwise elutions were made using 0.005M, 0.01M, 0.035M and 0.08M phosphate buffers of pH 8.0 and the fractions eluted with each buffer were pooled separately (Fraction I to IV). The protein eluted with 0.035M buffer (Fraction III) was again fractionated by gel filtration through a Sephadex G-200 column into three fractions (Fractions *III*₁, *III*₂, *III*₃) (Fig. 2). The six fractions (Fractions I, II, *III*₁₋₃, IV) were tested for the presence of γ A- and γ G-globulin by immunodiffusion with rabbit antisera specific for one of these immunoglobulins. Immunoelectrophoresis was also carried out using antihuman serum. It was found that Fractions I and II were composed of γ G-globulin and Fractions *III*₁, *III*₂, and IV contained both γ G- and γ A-globulins. Gamma G and α -globulins, but no γ A-globulin, were detected in Fraction *III*₃. Relative concentrations of γ G- and γ A-globulins in the fractions were determined by immunoplates containing either anti- γ G- or anti- γ A-globulin antibody, and the results are shown in Table III. The skin-sensitizing activity of the fractions was determined by P-K reactions. Fraction *III*₁, which had the highest concentration of γ A-globulin, showed the highest skin-sensitizing activity. However, the skin-sensitizing activity of the fractions did not parallel the γ A-globulin concentration.

The fractions containing skin-sensitizing antibody were absorbed with rabbit antibody specific for γ A- or γ G-globulin. Equal volumes of Fraction II (75 μ g N per milliliter) and the anti- γ G antibody preparation were mixed to remove γ G-globulin, and one-fifth volume of anti- γ A-globulin was added to one volume of Fraction II (100 μ g N per milliliter), although no precipitate was formed in the tube. An aliquot of Fraction *III*₁ (210 μ g N per milliliter) which contained 85 μ g N per milliliter of γ G-globulin was absorbed with an equal volume of anti- γ G-globulin. Another aliquot of Fraction *III*₁ (160 μ g N per milliliter) was mixed with an equal volume of anti- γ A-antibody. After 24 hours at 1 to 2° C., the precipitates were removed and the supernatants were tested for skin-sensitizing activity by P-K reactions, and for the presence of γ G- and γ A-globulins by immunodiffusion. The P-K reactions were repeated in three different individuals with comparable results (Table IV). The absorption of γ G-globulin from the fractions did not have a significant effect on the skin-sensitizing activity. The absorption of γ A-globulin from Fraction *III*₁ did not decrease the activity, whereas the activity in Fraction II diminished to half after addition of the antibody. These results are similar to those obtained with the fractions of the egg-sensitive patient's serum (cf. Table II). Since the absorption of either γ A- or γ G-globulin from Fraction *III*₁ was not accompanied by a decrease in skin-sensitizing activity, this fraction was absorbed with anti- γ A- and anti- γ G-globulin antibodies, successively. The proteins in the fraction were labeled with I¹³¹, and an aliquot of the sample was absorbed with anti- γ A at the equivalence zone, followed by the absorption with anti- γ G antibody. The final supernatant did not form a precipitin band with either antibody in Ouchterlony plates (Fig. 3). The absence of γ G- and γ A-globulin in the supernatant was

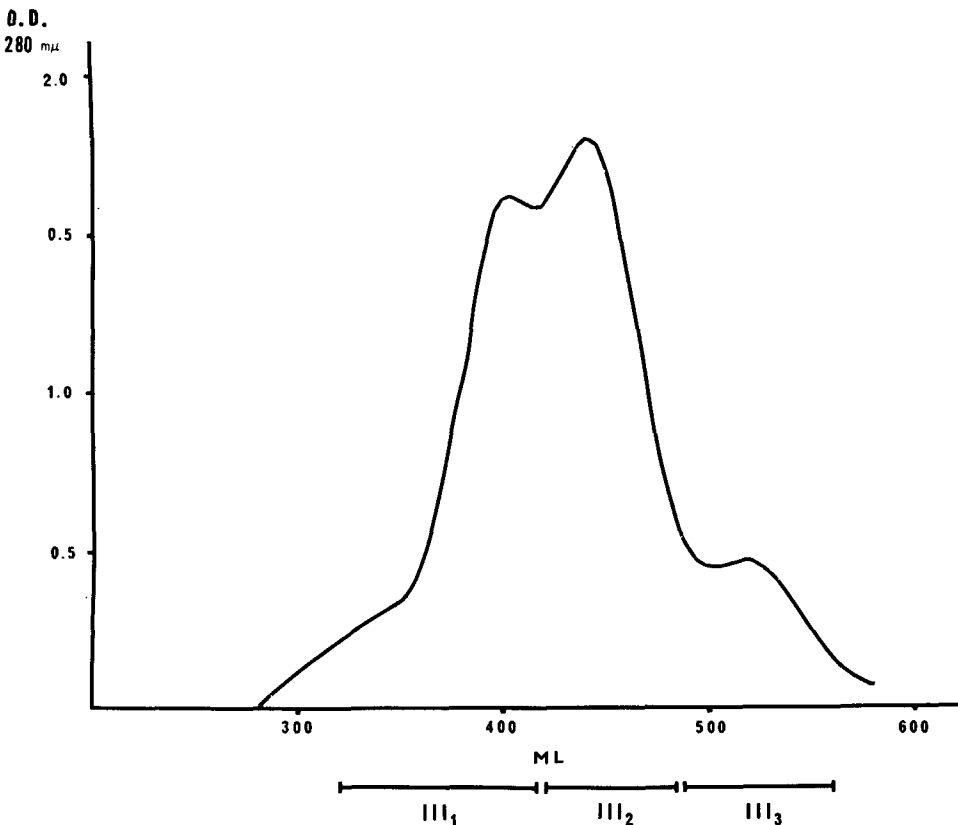


Fig. 2

Gel filtration of Fraction III of ragweed-sensitive serum through a Sephadex G-200 column (3.5 by 120 cm.).

Table III. Skin-sensitizing activity and γ -globulin concentrations in chromatographic fractions of a ragweed-sensitive patient's serum

Fraction	Phosphate buffer for elution*	Relative concentration of		Minimum skin-sensitizing dose (μ g N)		
		γ G (%)	γ A (%)	Total N†	γ G	γ A
I	0.005	100	0‡	5	5	—
II	0.01	100	0‡	0.1	ca 0.1	< 0.001
III ₁	0.035§	45	55	0.05	0.02	0.03
III ₂	0.035§	69	7.3	0.5	0.35	0.036
III ₃	0.035§	34	0‡	2	0.68	—
IV	0.08	9.4	7.7	5	0.45	0.4

*Stepwise elution from a DEAE cellulose column.

†The P-K reactions were carried out in two different normal individuals with comparable results.

‡No precipitin ring was formed on the immunoplate containing anti- γ A-globulin. The fractions containing 0.7 to 0.8 mg. N per milliliter gave a negative reaction with the anti- γ A-globulin in immunodiffusion in which 5 μ g N per milliliter of γ A-globulin can be detected.

§Fractions III₁₋₃ were obtained by gel filtration through a Sephadex G-200 column.

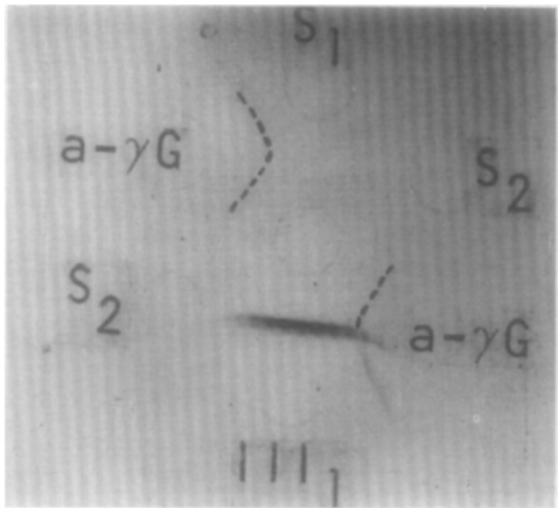


Fig. 3
Presence of γ A-globulin in Fraction III₁, before and after absorption with anti- γ A and anti- γ G antibodies. The antibody specific for γ A-globulin was placed in the center well. S_1 represents the supernatant obtained after the precipitation of γ A-globulin. S_2 represents the supernatant after the precipitation of γ A- and γ G-globulins. Faint bands were emphasized by dash lines in the photograph.

Table IV. *Effect of absorption with anti- γ -globulin on skin-sensitizing activity in fractions of a ragweed sensitive serum**

Frac-tions	Absorbed with	Presence of		Dilutions of fractions injected		
		γ G	γ A	$45 \times [0.8]$	$90 \times [0.2]$	$180 \times [0.1]$
II	Anti- γ G	-	-	40×52 (9.5)	20×23 (7)	13×21 (6.5)
	Anti- γ A	+	-	23×38 (8)	19×27 (7)	0
	—			40×44 (9)	23×48 (8.5)	17×33 (7)
III ₁				$100 \times [0.2]$	$200 \times [0.1]$	$400 \times [0.05]$
	Anti- γ G	-	+	27×40 (10)	27×45 (9)	25×38 (7)
	Anti- γ A	+	-	26×35 (9.5)	23×35 (8.5)	24×26 (7.5)
	Anti- γ A and anti- γ G	-	-	24×34 (9.5)	22×29 (8)	23×26 (7.5)
	—	+	+	27×43 (10.5)	30×32 (8.5)	26×37 (7.5)

*See legend for Table II.

confirmed by the interfacial ring test. Measurements of radioactivity showed that about 20 per cent of this activity remained in the supernatant after the absorption of γ G- and γ A-globulins. The skin-sensitizing activity of the supernatant was slightly less than that of the unabsorbed fraction (Table IV). Representative reactions with absorbed and unabsorbed preparations are shown in Fig. 4A. If one assumes that specific radioactivity is comparable for all proteins in Fraction III₁, as little as 0.01 μ g N of the protein in the supernatant gives a definite P-K reaction. Since the supernatant contained a large amount of rabbit

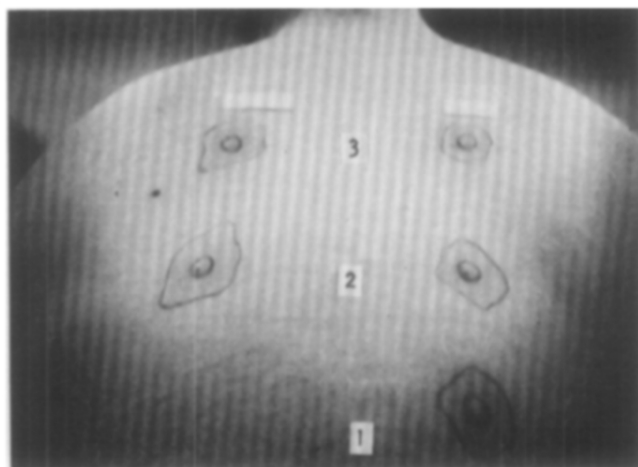


Fig. 4A

Prausnitz-Küstner reactions with a reagin-containing fraction (Fraction III, in Fig. 2) before and after the precipitation of γ A- and γ G-globulins with specific antibodies (cf. Fig. 3). Left, Skin sites 2 and 3 were sensitized with unabsorbed fraction containing $0.1 \mu\text{g N}$ and $0.05 \mu\text{g N}$, respectively. Right, The skin sites were sensitized with the supernatant after the precipitation of the immunoglobulins. The quantities of human serum protein injected, which were estimated from radioactivity, were $0.04 \mu\text{g N}$ (1), $0.02 \mu\text{g N}$ (2), and $0.01 \mu\text{g N}$ (3), respectively.

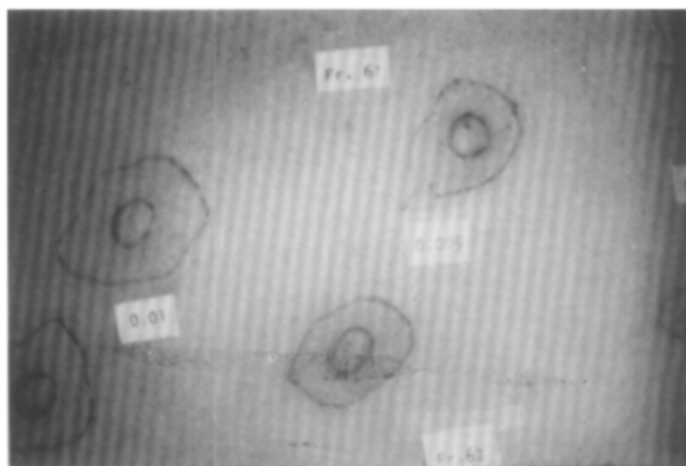
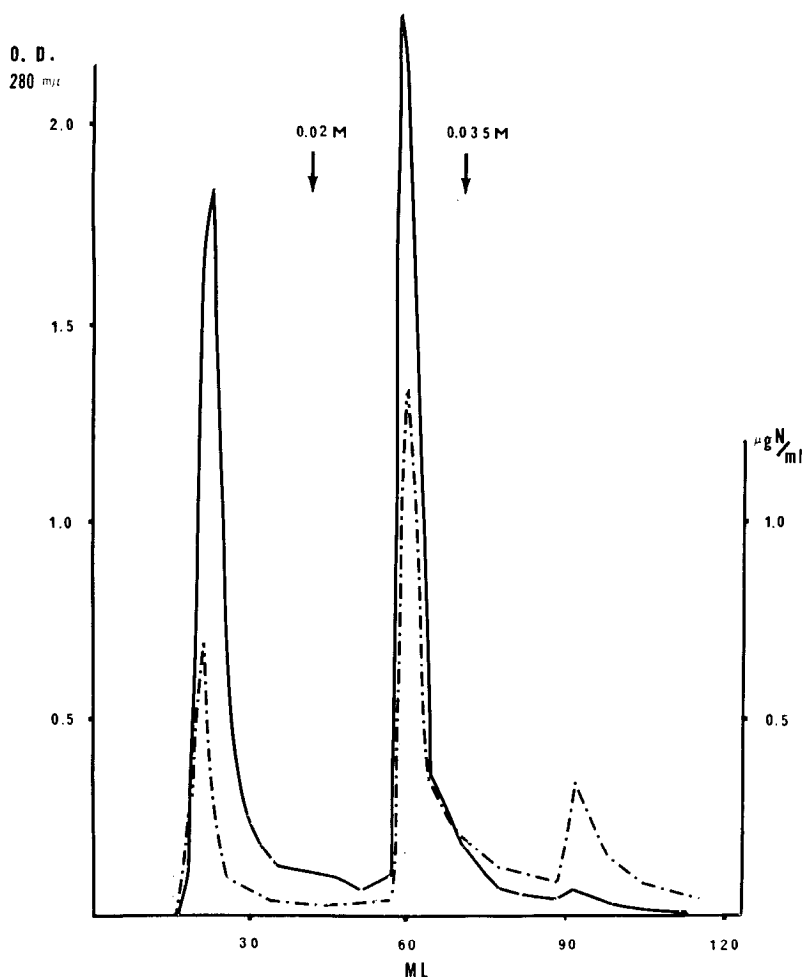


Fig. 4B

Prausnitz-Küstner reactions with Fractions 61 (90 to 91.5 ml.) and 62 (91.5 to 93 ml.) in Fig. 5. The quantity of human serum protein injected was estimated from radioactivity.

γ -globulin, it was fractionated by chromatography on a DEAE cellulose column. Three milliliters of the supernatant were dialyzed against 0.005M phosphate buffer (pH 8.0) and applied to a DEAE cellulose column (1 by 25 cm.) which had been equilibrated with the buffer. Stepwise elution was carried out using 0.005M , 0.02M , and 0.035M phosphate buffers (pH 8.0), and 1.5 ml. fractions

were collected. Absorption at 280 $m\mu$ and radioactivity of the fractions were determined. The results shown in Fig. 5 indicate that most of the protein was eluted with 0.005M and 0.02M phosphate buffers. Radioactivity measurements showed three peaks. Representative fractions of the three peaks were tested for skin-sensitizing activity. Fractions corresponding to the second and third peaks gave definite P-K reactions, whereas the three fractions from the first peak did not. In order to determine the minimal amount of protein required for the passive sensitization of human skin, serial dilutions of the fractions from the second and third peaks were injected intracutaneously into two normal individuals. The amount of human protein injected was estimated from radioactivity, assuming that all proteins in Fraction III₁ have comparable specific

**Fig. 5**

Rechromatography of the supernatant obtained after precipitation of γ G- and γ A-globulins from Fraction III₁ (c.f. Fig. 2). The solid line represents the absorption at 280 $m\mu$. The dotted line represents the concentration of human serum protein, which was estimated from radioactivity.

radioactivity. After 24 hours, the skin sites were challenged with 0.02 ml. of ragweed allergen containing of 10 PN units. In both normal individuals, the fractions containing 0.005 μg N of labeled protein gave positive reactions; only 0.0025 μg N of labeled protein in the peak of the third fraction was sufficient to sensitize their skin for a weak, but definite reaction (cf. Fig. 4B).

In order to study the nature of the protein with which the skin-sensitizing antibody is associated, the effect of absorption with the antibody specific for light chains on the skin-sensitizing activity was investigated. Fractions corresponding to the second (A) and third (B) peaks in Fig. 5 were pooled separately, and concentrated by negative pressure filtration. From radioactivity, the protein concentration in Fractions A and B was estimated to be 4.3 and 1.4 μg N per milliliter, respectively. Five-tenths milliliter of each fraction was mixed with 0.1 ml. of normal human γG -globulin containing 5 μg N and the antibody specific for light chains was added to each tube. The amount of the antibody added was four times as much as the equivalence amount for the carrier protein. After 24 hours at 1 to 2° C., the tubes were centrifuged in the cold and the precipitates washed three times with cold saline. The anti-light chain antibody but no γG -globulin was detected in the supernatants by interfacial ring tests. Radioactivity of the supernatants and precipitates was measured. It was found that 36.2 per cent and 21.7 per cent of the radioactivity in Fractions A and B coprecipitated with γG -globulin-anti-light chain complexes. Dilutions of the unabsorbed fractions and the supernatants were tested for the skin-sensitizing activity by P-K reactions. The amount of human serum protein injected for passive sensitization was estimated from the radioactivity. It was found that 0.005 μg of radioactive protein N in Fractions A and B gave definite P-K reactions, whereas 0.03 to 0.07 μg N in the supernatants did not (Table V). It is apparent that the reaginic antibody coprecipitated with γG -globulin-anti-light chain complexes.

DISCUSSION

The data presented in this paper clearly show that skin-sensitizing activity in the chromatographic fractions of reaginic sera does not parallel either γG - or γA -globulin concentration in the fractions. Relatively high reaginic activity was present in the fraction (Fraction II in Tables I and III) in which γA -globulin was not detected. From the sensitivity of interfacial ring tests and immunodiffusion, the γA -globulin concentration in the fraction of the ragweed-sensitive serum has to be less than 1 per cent of the total protein, if present at all. Since the fraction gave a definite P-K reaction with 0.1 μg N, the quantity of γA -globulin in the minimum skin-sensitizing dose of the fraction must be less than 0.001 μg N, whereas γA -globulin in the minimum skin-sensitizing dose of Fraction IV, which was eluted from the DEAE cellulose column with 0.08M phosphate buffer, was 0.4 μg N (Table III). Although no serum protein other than γG -globulin was detected in Fraction II by immunoelectrophoresis, complete absorption of γG -globulin from the fraction was not accompanied by any decrease in skin-sensitizing activity. It is apparent that the reaginic activity in the fraction is associated with a minor component(s) other than γG -globulin.

Table V. *Effect of absorption with anti-light chain on skin-sensitizing activity*

Frac- tion*	Absorp- tion†	Radioac- tivity‡ coprecipi- tated (%)	μg injected for passive sensitization‡				
			0.005	0.01	0.02	0.03	0.07
A	—	36.2	20 × 20 (7.5)	20 × 31 (9.5)	32 × 48 (9.5)	0	0
	+		nd	nd	nd		
B	—	21.7	20 × 24 (9)	28 × 33 (9.5)	nd	0	nd
	+		nd	nd	0		

Numerals in this table represent average diameters of erythema in millimeters; numerals in parentheses represent average diameter of wheal in P-K reactions in two normal individuals.

*cf. Fig. 5.

†Absorption with anti-light chain antibody was carried out using γG -globulin as a carrier. Radioactivity coprecipitated with the γG -globulin-anti-light chain antibody complexes was calculated (cf. text).

‡Estimated from radioactivity.

It was also found in the present experiments that the precipitation of γA -globulin in the fractions containing high reaginic activity resulted in either slight or essentially no decrease in skin-sensitizing activity. From the sensitivity of the interfacial ring test, over 95 per cent of the γA -globulin in Fraction III₁ of the ragweed-sensitive serum must be precipitated with the anti- γA -globulin antibody. However, the supernatant and the unabsorbed fractions were comparable with respect to the skin-sensitizing activity. The possibility remains that a part of γA -globulin may be in the form of soluble complexes with the anti- γA antibody and sensitized human skin. However, this possibility was excluded by the more recent experiments with γA -globulin fraction of anti-A human sera. The isoagglutinin activity in the fraction decreased to less than 1 per cent by the precipitation of the γA -globulin with the same preparation of rabbit anti- γA antibody. The slight decrease in skin-sensitizing activity after addition of the anti- γA -globulin to some other fractions, but not of anti- γG -globulin antibody, may be due either to the presence of γA -reaginic antibody or to the presence of large amounts of rabbit γ -globulin in the supernatant. Augustin and associates⁸ reported that rabbit γ -globulin blocked passive sensitization of human skin with reagin. The protein concentration in the anti- γA antibody preparation was about 3.5 times as high as the concentration in the anti- γG -globulin preparation. Nevertheless, our results showed the presence of reaginic antibody which was not precipitated with rabbit anti- γA -antibody. It was also found that the reaginic antibody was coprecipitated with γG -globulin-anti-light chain antibody complexes, indicating that the antibody molecules have light chain(s) of immunoglobulins.

The present experiments also showed the high sensitivity of P-K reactions. The fractions such as Fraction III of the egg-sensitive patient's serum and Fraction III₁ of ragweed-sensitive patient's serum gave P-K reactions with 0.02 to 0.025 μg N. In the experiment in which Fraction III₁ of the latter serum was labeled with I¹³¹, 80 per cent of the total radioactivity in the fraction was removed by the successive absorption with the antibodies specific for γA - and γG -globulins without significant decrease in skin-sensitizing activity (Table IV).

Furthermore, rechromatography of the supernatant after the absorption gave two fractions having high reaginic activity (Fig. 5). Assuming that the specific radioactivity was comparable for all proteins in Fraction III₁, as little as 0.0025 μg of protein N in one of the chromatographic fractions was sufficient to sensitize normal human skin for a positive P-K reaction. The addition of γG -globulin and anti-light chain antibody to these fractions resulted in the coprecipitation of 22 to 36 per cent of the radioactivity together with essentially all reaginic activity in the samples, indicating that the reaginic antibody was associated with the protein coprecipitated. As 0.005 μg N of the original unabsorbed fractions (Fractions A and B in Table V) gave positive P-K reactions, it seems that 0.001 to 0.0015 μg N of the protein contained an amount of reaginic antibody sufficient for sensitizing normal human skin.

The minimum quantity of reaginic antibody needed to produce a positive P-K reaction is not known. Stanworth²¹ has suggested that the minimum concentration of the antibody might be 0.02 μg N per milliliter. However, the present findings indicate that the quantity would be much less, because 0.001 to 0.002 μg N (0.02 to 0.04 μg per milliliter) of total protein in the reagin-containing fraction was sufficient for a positive P-K reaction. In our recent experiments on human antibody to type A blood group substance, the concentration of anti-A antibody in γG -, γA -, and γM -globulin fractions from type O individuals, who were immunized with A substance, was 0.2 to 2.3 per cent of the total protein.²² Furthermore, reaginic antibody may be inactivated during fractionation procedures. Stanworth²³ has reported that the reaginic antibody of active fractions of atopic patients' sera was inactivated by chromatography and ultrafiltration. Since the purification procedures in the present experiments involve chromatography and negative pressure filtration, the quantity of reaginic antibody in the active fractions may well be less than 1 per cent of the total protein. If this is the case, the minimum quantity of reaginic antibody required for a positive P-K reaction might be in the magnitude 10^{-5} μg N. It was found by Ovary²⁴ that the minimum quantity of guinea pig or rabbit antibodies for inducing passive cutaneous anaphylaxis (PCA) in guinea pigs is 0.003 to 0.01 μg N. It is evident that the P-K reactions in humans are much more sensitive than the PCA reactions in guinea pigs.

Concerning the physicochemical properties of reaginic antibody, a considerable body of evidence has been presented supporting the possibility that the antibody is associated with γA -globulin.⁴⁻⁹ One of the most important observations was reported by Fireman and co-workers,⁵ as well as Augustin and associates,⁸ who demonstrated that the reaginic antibody was precipitated with anti- γA -globulin antibody. The present results are quite different from their observations. The discrepancies between our data and theirs must await further studies, although several possibilities may be considered. One of the problems to consider in the physicochemical identification of reaginic antibody is its heterogeneity. Augustin and associates²⁵ and Fireman and associates⁵ obtained two to three reagin-containing fractions of patients' sera by chromatography on DEAE cellulose columns. In some cases, a major portion of reaginic activity was eluted from the column with buffers of 0.15M or higher molarity. Stanworth²³ obtained

a major component of reaginic antibody with a buffer of pH 6.1 and ionic strength 0.06. In our experiments and those reported by Humphrey and Porter,²⁶ the major portion of the activity was eluted with 0.01 to 0.035M buffers. The reaginic antibody studied in the present experiments is limited to the component eluted with the buffers of low ionic strength. The major part of the reaginic antibody studied by Fireman and associates⁵ was eluted with buffers of higher ionic strengths (0.05M phosphate and 0.1M NaCl). It is possible that the reaginic antibody studied in the present experiments is associated with an entirely different immunoglobulin from γ A- or γ G-globulin, whereas the antibody in Fireman's experiment may be γ A-globulin.

Another problem to consider is the difference between the anti- γ A-globulin antibody preparations used in the two experiments. Fireman and associates⁵ used a sheep antiserum and the absorption was carried out until the supernatant gave no precipitin reaction with the antiserum. In our experiments, γ A-globulin in the reagin-containing fractions was absorbed with rabbit antibody in the equivalence region. A possibility to consider in the classification of serum proteins is that the major antigenic determinants of human γ A-globulin molecules in sheep might be different from the antigenic determinants in the same molecules in rabbits. If this is the case, it is possible that the reaginic antibody may be associated with the protein which has the major γ A-globulin antigenic determinants in sheep but lacks the γ A determinant for rabbits. Augustin and associates⁸ used rabbit anti- γ A antibody for the absorption of the reaginic antibody. However, the antiserum used in their experiments showed two precipitin bands with human serum in immunoelectrophoresis.

The high sensitivity of the P-K reaction should be borne in mind for identification of the physicochemical nature of reagin. It is possible that the reaginic antibody detected in "pure γ A-globulin" in previous experiments^{8, 9} might be due to impurities which were not detected by usual immunochemical methods. Detection of minor components of serum proteins by immunodiffusion depends on the concentration of the antibody specific for the particular proteins. Our previous experiments⁸ indicated that normal human γ A-globulin blocked passive sensitization with reagin and supported the hypothesis that reaginic antibody is associated with γ A-globulin. These findings have to be criticized because the presence of a trace amount of minor components other than γ A-globulin in the normal γ A-globulin fraction cannot be excluded. In fact, the blocking activity of normal γ A-globulin was different depending on the samples used. In order to block passive sensitization with the minimal sensitizing dose of reaginic antibody, 0.5 to 5 μ g N of normal γ A-globulin was required. The possibility remains that the blocking of the passive sensitization is due either to impurities other than γ A-globulin or to a subclass of γ A-globulin in the preparations. The present experiments do not exclude the possibility that reaginic antibody in some patients may be associated with γ A-globulin, but indicate the presence of a reaginic antibody different from the bulk of γ A-globulin in antigenic structure. Recently, Rowe and Fahey^{27, 28} reported the presence of γ D-globulin in human sera in addition to γ G-, γ A-, and γ M-globulins. Experiments are planned to study the relation between the reaginic antibody and γ D-globulin.

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